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In Vitro and In Vivo Antagonism of Transforming Growth Factor-β Production by Interferon-α2b

by

Rajeet Singh Pannu

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Experimental Surgery

Department of Surgery

Edmonton, Alberta
Fall 1995

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of Canada

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Rajeet Singh Pannu
12620-66 Avenue
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" There is a square; there is an oblong. The players take the square and place it upon the oblong. They place it very accurately; they make a perfect dwelling place. Very little is left outside. The structure is now visible; what was inchoate is here stated; we are not so various or so mean; we have made oblongs and stood them upon squares. This is our triumph; this is our consolation."

- Virginia Woolf
The Waves

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled ***In Vitro and in Vivo Antagonism of Transforming growth factor-ß Production by Interferon-α2b*** submitted by ***Rajeet Singh Pannu*** in partial fulfillment of the requirements for the degree of Master of Science in Experimental Surgery.

Dr. Edward Tredget, Supervisor

Dr. Paul Scott, Committee Member

Dr. Aziz Ghahary, Committee Member

Dr. Steven Boyce, Committee Member

Dr. Ray Rajotte, Chairman

October 5, 1995

ABSTRACT

Hypertrophic Scarring (HSc) is a fibroproliferative disorder that often complicates the healing of burns or other injuries that penetrate the dermis. While generally not life threatening, HSc compromises the mobility of joints and is the source of significant morbidity in afflicted individuals. Though the etiology of HSc is not well established, the fibrogenic cytokine transforming growth factor-ß (TGF-ß) has been implicated as a possible causative agent.

Systemic administration of the cytokine interferon-α2b, in a phase I/II trial, to patients with HSc, resulted in significant clinical improvements. The interferons are a multifunctional cytokine family, that have been shown to have anti-fibrogenic effects on *in vitro* and *in vivo*. The hypothesis of this study is that the development of HSc is due to disregulation in the production of TGF-ß, and the antifibrogenic effects of IFN-α2b are due in part to its ability to antagonize the local and systemic production of TGF-ß.

A sandwich enzyme-liked immunosorbent assay was used to compare levels of TGF-ß in the serum of HSc patients to normal healthy age- and sex-matched individuals. It showed that TGF-ß levels are significantly higher in HSc patients, and reduce to normal levels over the course of treatment with IFN-α2b.

Secondly, TGF-ß levels were measured in the conditioned media of explanted and cultured HSc and normal skin fibroblasts of the same patient, with and without treatment with either IFN-α2b or IFN-γ. It was determined that HSc cells produced significantly more TGF-ß than normal cells. Treatment with IFN-α2b lowered HSc fibroblast TGF-ß production down to levels consistent with the normal controls.

This study correlated high levels of TGF-ß with HSc, and a reduction in TGF-ß levels with resolution of HSc. Furthermore, this study suggests that antagonism of TGF-ß production by IFN-α2b may be a means by which fibrosis can be controlled.

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For those who seek to learn, and by their toil
Open the portals of truth for all to see;
Offered to you is this, intact, unspoiled,
Low neither in content nor in honesty.
Seek and ye shall find!

After all is done, the work complete,
Let up your pace, give way, relax your feet.
Life is short.

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TABLE OF CONTENTS

CHAPTER 1 - GENERAL INTRODUCTION

A. Introduction....1
B. The Thesis....1
References....2

CHAPTER 2 - LITERATURE REVIEW

A. Introduction....3
B. Transforming growth factor-ß....4
C. The Interferons....26
D. Conclusion....35
References....36

CHAPTER 3 - ESTABLISHMENT OF A SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE QUANTITATION OF TGF-$ß_1$ IN HUMAN SERUM

Introduction....53
Materials and Methods....58
Results....60
Discussion....61
References....65

CHAPTER 4 - THE ROLE OF SYSTEMIC TGF-ß IN HYPERTROPHIC SCARRING POST-BURN: ANTAGONISM BY INTERFERON-α2b

Introduction....69
Materials and Methods....70
Results....73
Discussion....74
References....82

CHAPTER 5 - EFFECTS OF IFN-α2B AND IFN-γ ON TGF-ß PRODUCTION BY HYPERTROPHIC AND NORMAL DERMAL FIBROBLASTS *IN VITRO*

Introduction....84

CHAPTER 5 - continued

Materials and Methods....86
Results....89
Discussion....90
References....96

CHAPTER 6 - GENERAL DISCUSSION

A. Introduction....99
B. Synopsis of Results....99
D. Summary and Future Work....101
References....102

LIST OF TABLES

Table 3-1. Serum TGF-β1 levels in male blood donors.....62

Table 4-1. IFN-α2b Phase I/II Clinical Trial Patient Data.....76

Table 4-2.Serum TGF-β1 from HSc patients and Control Group.....77

Table 5-1. Cell strain data.....91

Table 5-2. Cell treatment data.....92

LIST OF FIGURES

Figure 3-1. Saturation kinetics for various TGF-β1 dilutions.....63

Figure 3-2. Regressed standard curve for TGF-β1.....64

Figure 4-1. Scatterplot of Control Group serum TGF-β1 levels.....78

Figure 4-2. Scatterplot of HSc patient and Control Group serum TGF-β1 levels.....79

Figure 4-3. Longitudinal study of serum TGF-β1 levels in one patient during treatment with IFN-α2b.....80

Figure 4-4. Scatterplots of HSc patient serum TGF-β1 levels over the course of IFN-α2b treatment.....81

Figure 5-1. TGF-β secretion by HSc fibroblasts and normal dermal fibroblasts.....93

Figure 5-2. Effect of IFN-α2b on TGF-β secretion by normal and HSc fibroblasts.....94

Figure 5-3. Effect of IFN-γ on TGF-β secretion by normal and HSc fibroblasts.....95

LIST OF ABBREVIATIONS

α2M	α2-macroglobulin
DMEM	Dulbecco's modified Eagles medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
FGF	fibroblast growth factor
HEPES	N-2-hydroxyethpiperazine-N'-2-ethanesulfonic acid
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
kb	kilobase
kD	kilodaltons
LAP	latency associated peptide
LTBP	latent TGF-ß binding protein
M6P	mannose-6-phosphate
mRNA	messenger ribonucleic acid
M.W.	molecular weight
NK	natural killer cells
O.D.	optical density
PBS	phosphate buffered saline
pg	picogram
S.E.M.	standard error of the mean
S.D.	standard deviation
TGF-ß	transforming growth factor-ß
TNF	tumor necrosis factor

CHAPTER 1 - GENERAL INTRODUCTION

A. INTRODUCTION

Hypertrophic scarring (HSc) is a dermal fibroproliferative disorder that causes significant morbidity and functional impairment in afflicted individuals (Scott et al., 1994). Though the etiology of this condition is not known, persistently high levels of transforming growth factor-ß (TGF-ß), a ubiquitously expressed fibrogenic cytokine, in HSc tissues suggests that it may play a role in the development of the disease (Ghahary et al., 1993; Scott et al., 1995). TGF-ß has been implicated in the development of numerous fibroproliferative disorders, and its antagonism *in vivo*, by the local or systemic administration of specific neutralizing molecules has proven effective in the management of different pathological conditions (Shah et al., 1992; Border et al., 1992).

B. THE THESIS

A phase I/II clinical trial of systemically administered IFN-α2b for the management of HSc has shown that it enhances the resolution and reduces the contraction and discomfort of HSc, while having relatively few side effects. The research in this thesis was conducted to determine the role of TGF-ß in the development of HSc, and to determine if the beneficial effects of IFN-α2b treatment may be related to direct or indirect antagonism of TGF-ß production.

Chapter Two is a literature review of the biology and biochemistry of TGF-ß and the interferons. Chapter Three describes the establishment of a sandwich enzyme-linked immunosorbent assay for the detection and quantitation of TGF-ß in biological fluids, based on the methodology of Danielpour (1992). In Chapter Four, the assay system is used to compare serum TGF-ß levels between HSc patients over the course of their treatment with systemically administered IFN-α2b, and a control population of age- and sex-matched healthy individuals. An *in vitro* cell culture system is used in Chapter Five, to determine the effects of the interferons on TGF-ß production by explanted HSc and normal dermal fibroblasts of the same patient. The final chapter is a synopsis and general discussion of the findings.

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CHAPTER 2 - LITERATURE REVIEW

A. INTRODUCTION

The ordered processes that result in wound healing are largely coordinated by a diverse group of soluble cytokines and growth factors. Produced by a wide variety of cells, these molecules can mediate both autocrine and paracrine signals, and act through multiple overlapping signal transduction pathways. While numerous growth factors are undoubtedly involved in wound healing, transforming growth factor-ß (TGF-ß) plays a particularly important role, and is considered fibrogenic for its dramatic effects on mesenchymal cell metabolism and extracellular matrix accumulation. It has also been implicated as a causative agent of wound healing disorders. Conversely, the interferon cytokine family (IFN-α, IFN-ß, IFN-γ) may be important regulators of wound healing. They are considered antifibrogenic factors, and may function by downregulating extracellular matrix synthesis and increasing matrix metabolism by specific gene regulatory mechanisms. In many cases an overabundance or a deficiency in the production of these molecules has been associated with pathological repair processes, therefore, a clear understanding of the complex interplay between synergizing and antagonizing cytokines may be important in the clinical management of wound healing disorders.

B. TRANSFORMING GROWTH FACTOR-ß

One of the major cytokines coordinating the interrelated processes of wound healing is TGF-ß. It is a member of a superfamily of soluble signaling molecules that plays a critical role in regulating cell proliferation and function. This growing group of related proteins includes the TGF-ß cytokine family, Müllerian inhibiting substance, the *decapentaplegic* gene product in *Drosophila* (DPP-C), *Xenopus* Vg-1, a family of bone morphogenic factors, and a family containing the activin and inhibin developmental proteins. These molecules share 23%-40% sequence identity in the C-terminal region including seven conserved cysteine residues, occur as secreted 25 kilodalton

(kD) disulfide-linked dimers, and bear no sequence homology to any other known growth factors (Roberts and Sporn, 1990).

There are at least five polypeptides (TGF-ß1-5) that are included in the TGF-ß family. They share between 70% and 80% sequence identity, and the sequences of the mature, proteolytically processed forms of each member is almost entirely conserved among species (Roberts and Sporn, 1990). In mammals only TGF-ß1, TGF-ß2, and TGF-ß3 have been found and their tissue specific differential expression may have important biological consequences. It is now well established that TGF-ß plays a fundamental regulatory role in development, osteogenesis, wound healing, immunomodulation and disease pathogenesis. While the signal transduction pathways initiated by TGF-ß have not been well elucidated, it is clear that TGF-ß is a multifunctional family of molecules that elicit cell and tissue specific responses and may work antagonistically or in conjunction with other matrix-bound and soluble proteins.

TGF-ß Genes

Given the multiple forms of the TGF-ß molecule and its presence in a large number of different organisms one might expect to find numerous gene sequence differences across species. This, however, is not the case. Cloning, sequencing, and characterization of the TGF-ß genes from different sources have demonstrated that they have very high sequence homology to the human TGF-ß isoforms. The human genes for the various isoforms are found on different chromosomes and encode unique transcripts. The TGF-ß1 gene in humans is found at 19q13 and encodes a 2.5 kilobase (kb) transcript, while the TGF-ß3 gene is located at 14q24 and encodes a 3.0 kb transcript. The gene for the TGF-ß2 isoform located in 1q41 is transcribed as 4.1 kb, 5.1 kb and 6.5 kb molecules that are thought to be processing intermediates of the same primary mRNA transcript (Roberts and Sporn, 1990).

The structure of the TGF-ß1 gene is also highly conserved among species. It is approximately 100 kb in length, contains seven exons, and the position of the splice junctions along the gene are highly conserved, being present in both bovine and porcine TGF-ß1 (Roberts et al., 1987). The same gene structure is also conserved in the TGF-ß2 and TGF-ß3 isoforms, with the exception of the first intron/exon junction which differs from TGF-ß1 by three

nucleotides. This suggests that the TGF-ß isoforms arose by duplication of a single ancestral gene. The primary gene transcripts are typically much longer than the actual 1200 nucleotide coding sequence, averaging several kilobases in length. This is due to distinctive 3' and 5' extension sequences that flank the coding region of the molecule. There is considerable variation among extension sequences, each isoform having a unique sequence of nucleotides (Nilsen-Hamilton, 1990; Roberts et al., 1987). The noncoding regions for TGF-ß1 are highly G-C rich while the TGF-ß2 extension sequences are A-T rich.

Sequencing data, deletional analysis, and DNAse I footprinting have demonstrated considerable variation and many unique features in the promoter regions of the gene isoforms. The TGF-ß1 promoter sequence extends 1400 base pairs (bp) upstream of the start site. It is G-C rich and contains several binding sites for transcription factors, including SP1, AP-1, and AP-2 (Kim et al., 1990). Interestingly, it has neither TATA nor CAAT box sequences. The promoter for the TGF-ß2 gene contains the TATA motif as well a CRE/ATF binding site for cyclic adenosine monophosphate (cAMP)-binding proteins (CREBs) (Roberts and Sporn, 1990). The TGF-ß3 promoter contains a CRE element and an SP1-binding site, and has been shown to be highly responsive to transcriptional upregulation by the SP-1 transcription factor (Geiser et al., 1993).

The variation among promoter regions clearly implies differential transcriptional regulation of the different isoforms. The reasons for differential expression of the isoforms are not understood, as the TGF-ß1 and TGF-ß2 isoforms are indistinguishable in biological assays (Roberts et al., 1987). Extracellular matrix proteins have been shown to be able to upregulate expression of TGF-ß1 and not TGF-ß2, indicating that the regulation of these genes is specific for the environmental context of the cell (Streuli et al., 1993). Furthermore, like many growth factors TGF-ß can upregulate its own expression in an autocrine fashion (Roberts and Sporn, 1990). This process may serve to amplify an initial signal and may play an important role in directing wound healing pathways and regulating the immune response. Current evidence suggests that the phorbol ester-induced AP-1 complex controls the TGF-ß1 autoregulatory pathway (Kim et al., 1990).

Alternate splicing of TGF-ß gene transcripts is a possible pathway by which TGF-ß may be regulated. In porcine tissues there is evidence of alternate splicing of TGF-ß1, as transcripts with an omission of exons 4 and 5

have been isolated (Roberts and Sporn, 1990). If the novel transcript were translated, the protein would have the same precursor N-terminal sequence as the native TGF-ß1 protein, but a different mature peptide sequence and possibly different functional properties. Alternate splicing of the TGF-ß genes has not been demonstrated in any other organisms and its functional significance is unknown.

TGF-ß Structure

a. General Structure

The structural features of the TGF-ß protein family are unique. Protein sequencing of the three mammalian TGF-ß isoforms has revealed that they have nine common cysteine residues and share the C-terminal sequence Cys-Lys-Cys-Ser. Each isoform is synthesized as a 390-412 amino acid precursor protein with a 4-5 amino acid processing site and a 20-23 amino acid signal peptide at its N-terminus (Derynck et al., 1985). After signal peptidase and proteolytic cleavage between amino acids 278 and 279, the precursor molecule is processed down to a 112 amino acid monomeric form that is termed the mature peptide (Wakefield et al., 1988). The biologically active structure of the molecule is a dimer of two TGF-ß molecules linked by a single disulfide bond at Cys77 of both monomers. While most TGF-ß isoforms exist as homodimers of two identical molecules, heterodimers of TGF-ß1 and 2 (termed TGF-ß1.2) and TGF-ß2 and 3 (termed TGF-ß2.3) have been isolated from porcine platelets and bovine bone respectively (Cheifetz et al., 1987; Ogawa et al., 1992). The biological activities and potencies of these molecules differ from those of the homodimers.

b. The Latency Complex

TGF-ß is secreted as a latent high molecular weight complex, consisting of a 25 kD dimeric mature peptide and the dimeric N-terminal precursor peptide or latency associated peptide (LAP). The LAP is part of the originally translated TGF-ß precursor peptide and consists of the remainder of the preproprotein minus the 23 amino acid N-terminal signal sequence and the C-terminal 112 amino acids of the mature TGF-ß molecule (Tsuji et al., 1990).

Analysis of the LAP-TGF-ß complex under reducing and non-reducing conditions shows that the LAP is glycosylated on asparagine residues, and contains mannose-6-phosphate (M6P) residues, and both a heparin- binding domain and an RGD recognition sequence for fibronectin and occurs in the latency complex as a disulfide-linked dimer of 77 kD (Ruoslahti and Pierschbacher, 1986; Wakefield et al., 1988). M6P is a lysosomal sorting marker that can be recognized extracellularly by the IGF-II receptor. It has been suggested that the presence of M6P may serve to target the TGF-ß precursor for extracellular activation (Dennis and Rifkin, 1991).

The LAP is sufficient to confer latency on TGF-ß. The configurations of the latency complex can differ depending on the source of the molecule. In cell types such as fibroblasts, platelets and bone cells, the latent TGF-ß complex also contains a protein termed the latent TGF-ß binding protein (LTBP). The LTBP is a 125-205 kD glycoprotein that associates covalently with the LAP (Dallas et al., 1994; Morén et al., 1994). It is not involved in maintaining latency though it may play a direct role in targeting and activating the latent complex. Recombinant TGF-ß produced by Chinese hamster ovary cells and TGF-ß produced by some osteoblast-like cells do not associate with an LTBP (Dallas et al., 1994). Fibroblasts and some osteoblasts produce a latent complex containing a 190 kD LTBP, while platelets produce a 130 kD truncated form of the same protein (Kanzaki et al., 1990; Wakefield et al., 1988). The possible functional significance of the various forms of the LTBP has not yet been established. Disruption of the latent complex, either by conformational changes induced by binding of another component, or by complete dissociation of the LAP, results in the activation of TGF-ß. Upon activation, TGF-ß can interact with any of several functionally unique cell surface receptors present on virtually all cell types, as well as numerous other membrane-bound, matrix and serum proteins.

c. Three Dimensional Structure

The recent 2.2Å and 2.1Å crystallographic resolutions of the three dimensional structure of the TGF-ß2 isoform have revealed unique folding features that are conserved in the gene superfamily. The TGF-ß2 monomer is a flat, elongated molecule with dimensions of 60 X 20 X 15$Å^3$. One end of the molecule is occupied by a long α-helix, perpendicular to a ß-sheet, and two

antiparallel ß-strands (Schlunegger and Grütter, 1992). Three intramolecular disulfide bridges connect the ß-strands to one another, while a fourth disulfide bridge connects an exposed amino terminal loop to the core of the molecule. Two of the ß-sheet disulfide bridges form a rigid eight-membered ring consisting of the residues Cys44-Ala45-Gly46-Ala47-Cys48-Cys111-Lys110-Cys109. This unusual structure is referred to as the TGF-ß knot. Similar eight membered rings have been found in endothelins, sarafatoxins, bee-venom toxins, and scorpion-venom toxins. The monomer has no hydrophobic core due to its extended structure, and has a solvent-accessible surface area of 4400 $Å^2$. In the dimer, two monomers are connected by a single disulfide bridge at Cys77. The solvent-accessible surface area is significantly reduced in the dimer, with about 1800 $Å^2$ buried within the molecule (Daopin et al., 1992). The general folding pattern of the TGF-ß2 molecule including the TGF-ß knot is thought to be the same for all the members of the TGF-ß family and similar for the gene superfamily due to high sequence identity among the molecules, particularly in regions containing disulfide-bonded cysteine residues (Archer et al., 1993).

Mutational studies done on the TGF-ß molecule suggest that it might exist in more than one conformation by reversible breakage of the Cys77 disulfide bond linking together the two TGF-ß monomers. Monomeric Cys77 to Ser77 mutants of TGF-ß1 exhibit interesting biological differences from the naturally-ocurring dimer (Amatayakul-Chantler et al., 1994). The mutant was 20% as effective as the wild-type dimer in promoting transcription of the plasminogen activator inhibitor-1 gene but was virtually unable to inhibit the growth of MvLu1 cells in culture. It also demonstrated preferential binding to a particular receptor class, the type I TGF-ß receptors. The unique nature and biological differences that the monomeric mutant exhibits, and the fact that it is stable and biologically active as a monomer, presents the formal possibility that it may actually occur as a native monomer with a free thiol group at Cys77. Though naturally occurring monomers have not been found, their existence is an intriguing possibility and would add a new dimension to TGF-ß structure/function study.

TGF-ß Receptors

Affinity-binding assays with [^{125}I]TGF-ß1 have identified three cell surface macromolecules that bind TGF-ß specifically. These structurally unrelated molecules were designated TGF-ß receptor types I, II and III, and have apparent molecular weights of 55 kD, 80 kD and 280 kD respectively (reviewed by Roberts and Sporn, 1990; Massagué, 1990; Lin and Lodish, 1993). Type IV, V and VI receptors have also been described though not functionally characterized. They are found as glycoproteins with molecular weights of 60 kD, 400 kD and 180kD respectively, and their tissue distributions appear to be highly specialized. The type I-III receptors are the most widely distributed membrane-bound TGF-ß binding proteins and are present on the surfaces of most TGF-ß-responsive cells (Segarini et al., 1989). They have high affinities for iodinated TGF-ß1, with dissociation constants of 5-25 picomolar (pM) for the type I and type II receptors, and 200 pM for the type III receptor. The affinity of each TGF-ß isoform for the surface receptors is different. The type III receptor seems to display equal affinity for the TGF-ß isoforms. The type I and II receptors have similar and much higher affinities for TGF-ß1 than for TGF-ß2. Though some of the structural features of these molecules have been elucidated, many of their functional properties have not been well characterized.

a. Type III Receptors

The type III receptor was considered originally to be the predominant TGF-ß signal transducing receptor due to its high level of expression in many cell membranes (Massagué, 1985; Cheifetz et al., 1987). It has since been identified as betaglycan, a non-signaling heparan sulfate/chondroitin sulfate proteoglycan with a core protein of 110-140 kD that exists both in soluble and cell associated forms (Andres et al., 1989). The rat betaglycan gene encodes an 853 amino acid protein with six N-linked glycosylation sites and at least one consensus site for glycosaminoglycan (GAG) attachment (Lopez-Casillas et al., 1991; Wang et al., 1991). Enzymatic removal of the GAG chains or release of the receptor from the cell surface does not alter the affinity of betaglycan for TGF-ß. The general structure of the molecule consists of a large extracellular GAG-linked N-terminal domain, a C-terminal membrane-

inserted domain, and a very short 41 amino acid cytoplasmic domain that may contain a phosphorylation site for protein kinase C but no known signaling motif (Gougos et al., 1992). The transmembrane and cytoplasmic domains of the molecule share 63% sequence identity to the corresponding regions of the endothelial TGF-ß-binding protein endoglin, while the extracellular domain contains regions of high sequence homology to the sperm receptors Zp2 and Zp3, the urinary protein uromodulin, and the zymogen granule membrane protein GP-2 (Bork and Sander, 1992). Mutational studies on betaglycan have localized the TGF-ß binding site to the first quarter of the extracellular domain in the juxtamembrane region (Fukushima et al., 1994). Interestingly, this area bears no similarity to any other TGF-ß binding proteins including endoglin.

b. Type II Receptors

The type II receptors are members of the serine/threonine receptor-kinases family that include the *C. elegans* daf-1 receptor and the activin receptor (Georgi et al., 1990; Mathews and Vale, 1991). The general structure of the type II receptor consists of a short cysteine-rich extracellular N-terminal domain followed by a single transmembrane segment and a long cytoplasmic C-terminal domain that is dominated by the kinase region of the molecule. A C-terminal extension sequence and a cytoplasmic spacer sequence that follows the transmembrane segment enclose the kinase domain of the molecule. Direct comparisons of known type II receptors show 40% amino acid identity in the kinase regions of the molecules. These receptors are also closely related to tyrosine kinases which has led some to speculate that they may be able to phosphorylate tyrosine residues as well (Lin and Lodish, 1993; Derynck, 1994).

c. Type I Receptors

Several type I receptors have been cloned and characterized. Like the type II receptors, these proteins are also transmembrane serine/threonine kinases. The presence of unique sequence features distinguish them from type II receptors. Their cytoplasmic domains are shorter and contain, following their kinase regions, a short C-terminal extension (Ebner et al., 1993). The

cytoplasmic juxtamembrane domain of the type I receptor contains a Ser-Gly-Ser-Gly-Ser-Gly-Leu-Pro motif termed the GS domain which is conserved in the type I receptor family and does not occur in type II receptors. The extracellular domains of these receptors are short, and contain a juxtamembrane cysteine cluster, and a distinct spacing of five conserved cysteine residues upstream from the transmembrane region.

d. Receptor Associations and Transmembrane Signaling

The TGF-ß receptors are able to associate with one another within biological membranes, though the significance of these observations is not clear. Mutational studies on Mv1Lu cells have demonstrated that type II receptors are able to bind ligands independently while the type I receptors require coexpression with the type II receptors for ligand binding (Wrana et al., 1994). Coimmunoprecipitation experiments further revealed that the type I and type II receptors form stable heterooligomeric associations and the type I, II and III receptors form stable homooligomeric associations, presumably dimers, within cell membranes in the absence or presence of biologically active TGF-ß (Chen and Derynck, 1994; Franzén et al., 1993). These experiments have not indicated the exact stoichiometry of these oligomeric complexes, the proportion of surface receptors that exist in these configurations, or the functional significance of the receptor complexes. Furthermore, experiments involving the expression of chimeric fusion constructs of the external and internal domains of the type I and type II receptors in mutant Mv1Lu epithelial cells have shown that direct interactions of the intracellular domains of these receptors is required for signal transduction. Interestingly, it was also observed that homomeric associations of type I receptors or the internal domains of type II receptors are unable to transduce signals in the presence of active TGF-ß (Okadome et al., 1994). The observed formation of these constitutive oligomeric complexes may resemble the insulin and IGF-1 signal transduction system where the receptors form homodimers in the absence of the active ligand (White and Kahn, 1994). Increased affinity of the type II receptor for TGF-ß when coexpressed with the type III receptor suggests that betaglycan may function by presenting ligand to signaling surface receptors and by acting as a membrane reservoir for TGF-ß (Lopez-Casillas et al., 1991). These hypotheses seem especially significant for the biological activity of

TGF-ß2 which has an order of magnitude lower affinity for type I and type II receptors than the TGF-ß1 isoform.

TGF-ß Signal Transduction

The direct signal transduction cascade initiated by the TGF-ß receptor system is not known at present (reviewed by Derynck, 1994). As TGF-ß is a growth inhibitor of a wide variety of cell types including epithelial cells and some immune cells, much of the work on TGF-ß signal transduction has focused on characterizing its growth inhibiting properties and examining its effects on cell-cycle regulatory components. Descriptive studies on TGF-ß-induced signal transduction have yielded several interesting observations. The addition of TGF-ß1 to exponentially growing cells results in the rapid expression of jun B, and the phosphorylation of nuclear protein (Pertovaara et al., 1989; Kramer et al., 1991). Furthermore, TGF-ß induces signaling through the ras and mitogen-activated protein kinase systems, which implies a close association of receptor stimulation with protooncogene-mediated signal transduction (Mulder and Morris, 1992). Phosphorylation of the cAMP response element binding protein is also a downstream consequence of Mv1Lu activation by TGF-ß, suggesting a role for G-proteins in the TGF-ß cascade (Kramer et al., 1991). Furthermore, the observation that in TGF-ß-treated cells that undergo growth arrest, the growth suppressor protein retinoblastoma (pRB) remains unphosphorylated and hence active in late G1 relates TGF-ß's effects to pRB (Laiho et al., 1990).

TGF-ß also appears to influence the activity of the G1 cyclin proteins through direct inhibition of cyclin-dependent kinase production. Cyclin-dependent kinases (CDK) are checkpoint control enzymes that are required for passage through specific intervals in the cell cycle. TGF-ß has been demonstrated to inhibit the production of CDK4, a protein required for G1-S transition (Ewen et al., 1993). This is followed by down regulation of the activation of CDK2 in Mv1Lu epithelial cells (Koff et al., 1993) implying a role for both CDK4 and CDK2 in TGF-ß induced growth arrest. Constitutive expression of CDK4 allows epithelial cells to overcome the effects of TGF-ß and progress into S phase (Ewen et al., 1993). Because CDK4 is thought to phosphorylate and inactivate pRB, inhibition of CDK4 production may link the two observations together. Though the timing and importance of these

events in the TGF-ß signaling pathway are not known, these observations certainly imply close associations between TGF-ß signaling receptors and critical cell cycle regulatory proteins.

TGF-ß Binding Molecules

In vitro and *in vivo* studies have demonstrated that while most cells express surface receptors for TGF-ß, its biological activities are tightly regulated and often specific for individual cell and tissue types. One of the possible mechanisms for this control is by association of biologically active TGF-ß with soluble or extracellular matrix (ECM)-associated non-receptor binding molecules. These associations could serve to protect it from proteolytic degradation, limit its activity by competing with surface receptors, localize it to specific areas such as wound sites for future activity, or ensure its transient activation and rapid inactivation. A large number of macromolecules have demonstrated binding affinities for TGF-ß, and local concentrations of these factors may play critical roles in modulating its function. It is currently thought that the serum protein α2-macroglobulin (α2M), the polyanionic carbohydrates heparin and fucoidan, the multifunctional protein thrombospondin, and the matrix proteoglycan decorin, are able to bind TGF-ß and may influence its biological activities.

a. α2-Macroglobulin

α2-macroglobulin is a major circulating inhibitor of a wide variety of serum endopeptidases. When a susceptible peptide bond is cleaved on its surface, α2M undergoes a rapid conformational change causing it to bind and sterically inhibit an attacking enzyme (Tsuji et al., 1990). Serum α2M is often found in covalent and non-covalent associations with TGF-ß with a stoichiometry of two TGF-ß molecules per α2M tetramer. The ability of α2M to inactivate proteases and its ability to associate with TGF-ß may occur by common mechanisms. Though TGF-ß is latent when associated with α2M, the TGF-ß latency proteins are not found in the complex. Interestingly, latent TGF-ß containing the latency proteins does not bind α2M, but upon activation by transient acidification gains this ability (Tsuji et al., 1990). These observations suggest a possible role for α2M as a regulatory scavenging

protein that binds and neutralizes biologically active TGF-ß (Phan et al., 1989; Tsuji et al., 1990). The fact that most of the TGF-ß found in serum is covalently associated with α2M and unable to be activated by transient acidification or proteolysis supports this hypothesis (McCaffery et al., 1992).

b. Polyanionic Carbohydrates

The ability of growth factors to bind carbohydrates may have important biological consequences. Like many other cytokines including granulocyte macrophage colony stimulating factor and the fibroblast growth factors, TGF-ß is able to associate with polyanionic carbohydrates. The interactions of the polysaccharides heparin, heparan sulfate, fucoidan, and polyinosinic acid with TGF-ß appear to be specific and not exclusively charge-dependent, as the equally charged molecules chondroitin sulfate and polycytidylic acid show no ability to associate with TGF-ß (McCaffery et al., 1994). Both heparin and fucoidan, a carbohydrate isolated from the seaweed *fucus vesiculosus*, can protect TGF-ß from proteolyic degradation, enhance its bioactivity, and inhibit the ability of α2M to bind and inactivate TGF-ß *in vitro*. Interestingly, affinity binding experiments with synthetic peptide fragments of the TGF-ß heparin-binding region have shown that not all heparin fragments are able to form such associations (McCaffery et al., 1992). This implies both structural heterogeneity and functional diversity of heparin molecules. These observations suggest that the glycosaminoglycan heparin and possibly other carbohydrates may function by antagonizing TGF-ß-neutralizing molecules, augmenting its biological effects, and enhancing it biological availability.

c. Thrombospondin

The recent demonstration that the ECM protein thrombospondin can bind and activate TGF-ß *in vitro* has changed some of the previously held notions concerning TGF-ß latency. Thrombospondins (TSPs) are a family of multidomain glycoproteins that are secreted by a variety of normal and transformed cells in culture. They are present in platelet α-granules, wound fluids, embryonic and connective tissues (Schultz-Cherry et al., 1994a). TSPs are able to interact with a large number of macromolecules, including collagens, heparin, plasminogen activating factor, secreted protein acidic rich

in cysteine (SPARC), and fibronectin (Lahav, 1993). These molecules fall into roughly three categories: ECM components, proteins involved in the complement cascade, and molecules that mediate TSP interactions with cell surfaces. Unlike many matrix proteins, TSPs are functional signaling molecules that are able to influence cell behaviour much in the manner of cytokines. They can stimulate fibroblast proliferation, smooth muscle cell growth in synergy with epidermal growth factor (EGF), and inhibit angiogenesis and endothelial cell growth (Lahav, 1993; Phan et al., 1989). The prototypical member of the thrombospondin family is TSP1. TSP1 occurs as a trimer and has several functional domains. It has a 25 kD heparin-binding domain at its N-terminal, two cysteine residues at positions 252 and 256 that form disulfide bridges linking the trimer together, a 90 amino acid procollagen-like domain that may be responsible for its ability to bind collagen, and three 60 residue properidin-like repeats each of which contains six conserved cysteines. These "type I" repeats have 47% sequence identity to the complement protein properidin. There are also three EGF-like repeat sequences termed type II repeats, and eight calcium-binding type III repeat sequences. Fibronectin-binding RGD sequences are also found within the type III region (Schultz-Cherry et al., 1994b). TSP1 complexes with latent TGF-ß in solution (Schultz-Cherry et al.,1994a). The precise mechanism of this association is unknown, but antibodies to the N-terminal region of the LAP inhibit TSP1 binding. The presence of the LTBP in the TGF-ß latency complex does not appear to affect its ability to bind TSP1. While latent TGF-ß is unable to bind cell surface receptors, the binding of TSP1 activates the latent cytokine. Interestingly, thrombospondin does not appear to cause the dissociation of the LAP, rather crosslinking studies have shown that it activates TGF-ß simply by binding the latency complex (Schultz-Cherry et al., 1994a). Though it had previously been thought that complete dissociation of the LAP was necessary for TGF-ß activation, either reversibly by transient acidification or irreversibly by proteolysis or heat treatment, these experiments have shown that conformational changes in the latency complex may unmask cryptic structures and activate the protein. This is further demonstrated by the fact that antibodies to biologically active TGF-ß bind the TSP1-TGF-ß complex. The binding site for TGF-ß on TSP1 may be the properidin-like type I repeats. Bacterially expressed fusion constructs of various domains of TSP1 have shown that only the type I region is able to bind and activate latent TGF-ß.

As well, antibodies to the type I repeats inhibit the ability of TSP1 to bind and activate the cytokine (Schultz-Cherry et al., 1994b). There may be an important biological significance for TSP1-TGF-ß1 complexes. Because both molecules are present in platelet α-granules and have been found in complexes upon platelet degranulation, it is conceivable that TGF-ß is in fact biologically active when released during the clotting process.

d. Decorin

One of the most intriguing and in some ways perplexing TGF-ß binding molecules is the small interstitial proteolycan decorin. Decorin is a dermatan sulfate/chondroitin sulfate proteoglycan with a globular core protein that associates with collagen in the extracellular matrices of many tissues (Kresse et al., 1993). It carries a single N-terminal glycosaminoglycan chain that is linked to Ser4 of the mature core protein. Some suggest that the unique structural features of decorin allow it to bind TGF-ß with specificity. Almost 80% of the molecule consists of ten repeats of a leucine-rich sequence of about 24 amino acids, a motif that is found in the proteoglycans biglycan and fibromodulin, and other unrelated proteins such as the *Drosophila* morphogenic proteins. Interestingly, biglycan and fibromodulin are also able to associate with TGF-ß *in vitro*, which may indicate a functional importance for these repeat sequences in growth factor binding (Hildebrand et al., 1994). Several observations have identified decorin's possible role in modulating TGF-ß activity. Affinity chromatography experiments have shown that decorin can associate with TGF-ß. Furthermore, decorin-TGF-ß complexes were found to be biologically inactive in Mv1Lu epithelial cell bioassays, suggesting that decorin binding may inhibit the ability of TGF-ß to associate with its receptors (Yamaguchi et al., 1990). The fact that bacterially produced recombinant decorin core protein and decorin isolated from mammalian connective tissues both bind TGF-ß suggests that the core protein and not the GAG chains are responsible for the observed phenomenon. In an *in vivo* model of TGF-ß-induced experimental glomerulonephritis in rats, intravenous administration of both recombinant and purified decorin was able to inhibit glomerular matrix accumulation and reverse the pathological manifestations of the disease (Border et al., 1992). Because the ECM accumulation observed in experimental glomerulonephritis is thought to be caused by TGF-ß, these

experiments seem to indicate that decorin has an ability to inhibit TGF-ß activity *in vivo*. However, when tested in other *in vitro* assay systems such as collagen gel contraction and assays for TGF-ß-induced upregulation of biglycan gene expression in osteosarcoma cells, decorin was unable to counteract the effects of TGF-ß (Hausser et al., 1994). These findings suggest that decorin is able to inhibit only certain specific actions of the cytokine. Paradoxically, decorin has also been shown to enhance the activity of TGF-ß. Decorin derived from bone matrix proteoglycans has been shown to augment TGF-ßs ability to inhibit the growth of osteoblast-like MC3T3 cells (Takeuchi et al., 1994). In the same assay system it was observed that decorin was also able to enhance the binding affinity of TGF-ß to membrane bound receptors. Because the data concerning the biological consequences of TGF-ß-decorin association is contradictory and the signal transduction pathways of TGF-ß have not yet been determined, it is difficult to speculate on the actual inhibitory mechanisms of decorin. It may be that decorin prevents TGF-ß from associating with a specific membrane receptor complex, while having no effect on other types of receptor-ligand interactions, which would account for its differential effects in different systems. One might also speculate that the presence of other interacting molecules in a system may influence the ability of decorin to associate with TGF-ß. The observation that both the protein core molecule and the glycosaminoglycan chains of decorin are able to bind thrombospondin, a known TGF-ß activating protein, support this notion, though the importance of this association with respect to its effect on TGF-ß activation is unknown (Kresse et al., 1993). While much of the data on TGF-ß-decorin complexes is derived from studies of soluble phase interactions between the two molecules, *in vitro* evidence indicates that decorin in tissue is found in association with collagen fibrils and not free in solution (Kresse et al., 1993). The ability of collagen-bound decorin to associate with growth factors may be much different from that of free decorin due to structural alterations in the protein.

Though the importance of the various TGF-ß binding molecules is not yet fully understood, it is clear that the actions of TGF-ß are highly regulated. Circulating binding molecules, carbohydrates, and matrix components form associations with the cytokine *in vitro* and in the case of decorin may antagonize its effects *in vivo* as well. The functions of TGF-ß seem to be modulated by the ECM. The large number of tissue components that appear

to associate specifically with TGF-ß demonstrates that the complexity of its regulation extends beyond its production and degradation. Extracellular matrix molecules by modulating the actions of signaling proteins are critical components of the immune system and may play a role in disease pathogenesis.

Cellular Effects

TGF-ß is a multifunctional cytokine that influences many different physiological processes through a variety of different cell types. The nature of its actions on target cells are dependent on a number of parameters including the cell origin and its state of differentiation, local concentrations of various activating and inhibiting molecules, the presence of other growth factors, its structure within the latency complex, the architecture of the ECM, and the availability of cell surface receptors. Its most significant effects on cultured cells *in vitro* occur on mesenchymal cells, epithelial cells, and hematopoetic cells which suggests a number of different roles for the cytokine *in vivo*. The ability of TGF-ß to function in different environmental contexts provides clues for its physiological roles and its possible involvement in the causation of disease.

a. Mesenchymal Cells

By influencing the growth and metabolism of mesenchymal cells, TGF-ß's major physiological function seems to be regulating the accumulation of and response of cells to components of the ECM (reviewed by Roberts and Sporn, 1990). TGF-ß has been shown to activate gene transcription of matrix components and protease inhibitors, decrease synthesis of proteolytic enzymes that degrade ECM proteins, increase production of cellular receptors for matrix proteins, and influence mesenchymal cell growth and differentiation. Currently it is thought that the biosynthesis and degradation of a large number of matrix and matrix associated components is controlled in some fashion by TGF-ß. TGF-ß increases mesenchymal cell production of type I, III, IV and V collagens, thrombospondin, certain dermatan sulfate/chondroitin sulfate proteoglycans, and the SPARC glycoprotein (Rossi et al., 1988; Madri et al., 1988; Pentinnen et al., 1988, Westergren-Thorsson et al., 1991; Reed et

al., 1994). The isoforms TGF-ß1 and TGF-ß2 seem to be equipotent in eliciting these effects. The ability of TGF-ß to influence matrix production and accumulation may occur by several mechanisms. Nuclear run-on experiments have suggested that increases in types I, III and V collagen and fibronectin mRNA expression may occur by stabilization of the transcript and not by up regulation of the genes (Ignotz et al., 1987). Other studies have shown that TGF-ß can directly enhance the transcription of the mouse α2(I) collagen promoter and the fibronectin promoter, possibly through the NF-1 (nuclear factor-1) transcription factor (Rossi et al., 1988; Dean et al., 1988). TGF-ß also appears to influence matrix accumulation by inhibiting the production of matrix-degrading proteases, and increasing the production of protease inhibitors. Culture studies have demonstrated that production of thiol protease, serine protease, plasminogen activator, collagenase, elastase, cathepsin L and transin/stromelysin is down regulated by TGF-ß (Chiang and Nilsen-Hamilton, 1986; Laiho et al., 1990; Lund et al., 1986; Overall et al., 1989; Matrisian et al., 1986). Furthermore, the matrix protease inhibitors plasminogen activator inhibitor and tissue inhibitor of metalloproteases (TIMP) are upregulated by TGF-ß (Edwards et al., 1987).

Interestingly, many cellular responses elicited by TGF-ß may occur through cooperative and antagonistic interactions with other cytokines. The reciprocal effects of TGF-ß on the production of TIMP and collagenase have been shown to require bFGF and EGF (Edwards et al., 1987). The addition of TGF-ß alone to cultured fibroblasts has no effect on the production of TIMP or collagenase, while in the presence of EGF and bFGF, TGF-ß-induced transcriptional regulation of these molecules was observed. Similarly, the actions of other signaling molecules may occur through TGF-ß. The ability of the hormone angiotensin II to stimulate ECM production in rat glomerular mesangial cells has been shown to be mediated by TGF-ß *in vitro* (Kagami et al., 1994). There is also evidence of TGF-ß paracrine loops within tissues resulting in the modification of mesenchymal matrix production. Mast cells stimulated through their FcεRI receptors produce TGF-ß and TNF-α. Co-culture of these stimulated mast cells with mouse dermal fibroblasts demonstrated that in the presence of activated mast cell medium, stimulation of mouse fibroblast collagen production was observed (Gordon and Galli, 1994). Regulation of TGF-ß seems to occur at some level through complex interplay

with other growth factors, and therefore local concentrations of different cytokines may have significant influence on TGF-ß's functions.

TGF-ß also alters the behavior of mesenchymal cells. The ability of fibroblasts to contract collagen gels, a process mediated by cell surface integrins, is strongly enhanced by TGF-ß (Montesano and Orci, 1988). Correspondingly, it has also been shown that TGF-ß increases integrin synthesis, and differentially regulates the production of the individual integrin subunits by different mechanisms (Roberts and Sporn, 1990). While the role of TGF-ß in stimulating mesenchymal ECM production *in vitro* is clear, the ability of TGF-ß to influence mesenchymal cell growth is at present controversial. TGF-ß was initially characterized for its ability to cause anchorage-independent growth of normal rat kidney fibroblasts on soft agar (Roberts and Sporn, 1990). It has since been shown that TGF-ß stimulates DNA synthesis in fibroblasts, a process which may be mediated by platelet-derived growth factor (PDGF). Paradoxically, some studies have shown that when applied to cultured dermal fibroblasts, TGF-ß inhibits proliferation unless added in conjunction with EGF (Fukami et al., 1995). The *in vitro* evidence therefore supports the notion that TGF-ß specifically influences the ability of mesenchymal cells to produce components of the ECM. While the *in vivo* effects of TGF-ß may be more complex, there is little doubt that TGF-ß is a critical regulator of mesenchymal cell function and is involved in developmental and tissue repair processes.

b. Epithelial Cells

TGF-ß is also a potent *in vitro* growth inhibitor of a variety of epithelial cells, including hepatocytes, keratinocytes, bronchial epithelial cells, intestinal epithelial cells, and renal proximal tubular cells (Roberts and Sporn, 1990). Because growth and differentiation are closely linked processes in epithelia, TGF-ß-induced growth inhibition is coincident with upregulation of some epithelial terminal differentiation markers. TGF-ß also causes reversible growth inhibition of keratinocytes in culture, but, paradoxically is unable to delay re-epithelialization in either organotypic *in vitro* wound models or *in vivo* models of wound healing (Garlick and Taichman, 1994). Keratinocytes express TGF-ß mRNA and protein which suggests that the cytokine may be a autocrine growth regulator in the skin (Coffee et al, 1988; Kane et al, 1991) .

This is supported by the observation that treatment of mouse skin with the tumor promoting phorbol ester 12-0-tetradecanoylphorbol-13-acetate causes a rapid increase in TGF-ß expression in the epidermis, which suggests a growth control biofeedback mechanism (Eshrick et al, 1993).

c. Immune Cells

While exhibiting similar actions on most other cell types, ie. regulating their growth and/or ability to produce ECM proteins, TGF-ß functions in a unique and different manner on hematopoetic immune cells. TGF-ß is a potent immunomodulatory protein that may be critical to selective immunosuppression as well as immune class regulation. It has been observed that TGF-ß suppresses the proliferation and function of both B and T lymphocytes while activating and augmenting macrophage function (Wahl, 1992). Furthermore, TGF-ß is known to inhibit the proliferation of thymocytes and the activity of natural killer (NK) cells, while modulating the proliferation of lymphokine-activated killer (LAK) cells and allospecific cytotoxic T lymphocytes without impairing their function (Wahl, 1992; Rook et al., 1986). TGF-ß therefore appears to function by selecting specific immune pathways and inhibiting others.

Immune cell function is in many ways determined by the production and activity of specific cytokine cascades that coordinate an immune response. One of the most important functions of TGF-ß seems to be to antagonize or synergize with these immune signals and thereby regulate immune cell function. Its ability to influence cell function in the presence of other cytokines is possible because many immune cells exhibit exquisite sensitivity to TGF-ß and are able to respond to fentomolar concentrations. It is able to antagonize the effects of a number of different cytokines in specific situations, including interleukins (IL)-1, IL-2 and IL-3, interferon-α, interferon-γ, and TNF-α (Wahl, 1992). The effects of TGF-ß on interferon-γ function are particularly interesting because of the important role interferon-γ plays as a principle mediator of immune class regulation. Among its many functions, interferon-γ causes the expression of MHC class II antigens on the surface of both lymphoid and non-lymphoid cells during the induction of the cell-mediated machinery. TGF-ß is able to inhibit this process *in vitro* suggesting that it may play a role in immune class switching. There is also evidence that

TGF-ß is involved in antibody isotype switching. TGF-ß is able to inhibit IgG and IgM production by B cells, and when added in conjunction with IL-2 and IL-5, TGF-ß upregulates IgA secretion by splenic lymphocytes (Wahl, 1992). The role that TGF-ß may play in lymphoid IgA production, however, is still unknown. Paradoxically, TGF-ß, when added to IgA producing plasma cells, inhibits production, implying that its major role may be to stimulate immune cell differentiation. While for some types of T lymphocytes TGF-ß is an inhibitor, other types of effector T cells are rapidly activated by TGF-ß, proliferate vigorously and produce large amounts of IL-2 (Cerwenka et al., 1994).

TGF-ß Physiology and Pathophysiology

a. Immunosuppressive Effects

The immediate release of TGF-ß by degranulating platelets at sites of immunologic challenge or injury suggests that it plays an important role in the inflammatory cascade. TGF-ß seems to have both pro-inflammatory and anti-inflammatory effects *in vivo*. When introduced exogenously into tissue, TGF-ß is a chemoattractant for monocytes, T-cells, and neutrophils (Wahl and Wahl, 1992; Fava et al., 1991, Adams et al., 1991). Though it has been shown to inhibit the binding of lymphocytes to endothelial cells *in vitro*, the ability of TGF-ß to up regulate integrin production by immune cells suggests that it may play a role in facilitating tight associations between rolling lymphocytes and the vascular bed (Roberts and Sporn, 1990). TGF-ß also increases monocyte production of gelatinase and type IV collagenase, enzymes which aid in degrading the basement membrane and allow for diapedesis of circulating immune cells (Wahl, 1994). While these facts seem to indicate that TGF-ß may enhance inflammation, there is abundant evidence to suggest that it is also an important inhibitor.

The most convincing evidence of its immunosuppressive role comes from studies done on TGF-ß "knockout" mice. The murine TGF-ß1 gene was disrupted in embryonic stem cells by homologous recombination, and inbreeding of heterozygous mice was done to generate TGF-ß1 (-/-) offspring (Kulkarni et al., 1995; Shull et al., 1992). Only forty percent of the null mice came to term, while sixty percent died in utero. The surviving mutant mice

showed no developmental abnormalities, though they all succumbed to a lethal multifocal inflammatory disease approximately two weeks post-partum (Shull et al., 1992). Surprisingly, the presence of TGF-ß1 in the tissues of the mice was identified by immunohistochemistry in the absence of gene expression. It was later determined that maternal transfer of the cytokine was responsible for this finding and reduction of the maternal TGF-ß supply seemed to coincide with the onset of the inflammatory condition (Letterio et al., 1994). Pathological analysis of the mice demonstrated severe inflammation of heart, lungs, thymus, stomach, colon, pancreas and liver (Kulkarni et al., 1995). Death was ultimately attributed to cardiopulmonary failure. Eight days post partum, it was noticed that there was a dramatic increase in leukocyte adhesion to the luminal side of the vascular endothelium of pulmonary veins and venules. Furthermore, there was increased expression of MHC I and II proteins in multiple tissues of the mutant mice which may have predisposed them to the autoimmune disease. The T lymphocytes of the null mice were abnormally activated, characterized by hyperproliferation and spontaneous overproduction of IL-2 and TNF-α (Christ et al., 1994). Thus the TGF-ß null mouse model suggests that one of the most important roles that the molecule plays *in vivo* is as a regulator of the inflammatory cascade.

Much pathophysiological data links TGF-ß with immunosuppression in disease as well. The association of TGF-ß and acquired immune deficiency syndrome (AIDS) is thus not suprising. *In vitro* infection of peripheral blood mononuclear cells with the HIV-1 virus causes increased production of TGF-ß and IL-6 (Allen et al., 1991; Nakajima et al., 1989). Also, TGF-ß has been shown to enhance replication of the virus in PHA-activated mononuclear cells (Lazdins et al., 1991). The role of TGF-ß in suppressing immune function may facilitate the survival of pathogens in tissues. Experimental models of *Trypanosoma cruzi* infection show that systemically administered TGF-ß augmented the ability of the parasite to produce lethal infections (Silva et al., 1991). Furthermore, MRL/lpr mice, which are susceptible to systemic autoimmune disorders due to defective neutrophil function, produce higher levels of TGF-ß than mice without the deficiency, and are also more susceptible to infection by gram positive and gram negative bacteria (Lowrance et al., 1994). The subsequent injection of anti-TGF-ß neutralizing antibodies significantly ameliorated the host defense defect of the mice, demonstrating that overproduction of TGF-ß was responsible for the observed

immunosuppression. Impaired immune response in hemophiliacs may also be associated with TGF-ß. Hemophiliacs often suffer from deficient lymphoproliferative responses, decreased IL-2 production by immune cells, and NK cell and B cell dysfunction (Wadhwa et al., 1994). The observation that TGF-ß is a contaminant in factor VIII concentrates may at least partially explain the immunosuppression observed in recipients of the blood product. Hemorrhage associated with trauma is another pathological situation that is characterized by impaired immune function. Studies have demonstrated that hemorrhage induces profound depression of splenocyte and macrophage function that may be associated with increased levels of TGF-ß production (Ayala et al., 1993). Plasma levels of TGF-ß in a mouse hemorrhage-model were significantly upregulated compared to control animals up to 72 hours post-induction, suggesting that hemorrhage induces a sustained release of TGF-ß into blood. Furthermore, when these animals were treated with anti-TGF-ß neutralizing antibodies there was significant and specific downregulation of TGF-ß production which may indicate autocrine control of TGF-ß release.

The mechanisms by which TGF-ß modulates the immune system are not clear, though its actions as both immunoactivator and immunosuppressor seem contradictory. Current speculation is that TGF-ß may function differently depending on the context of its activation. Local production of TGF-ß might provide a chemotactic gradient that attracts immune cells, as evidenced in tissue injection studies, while systemic administration may destroy the gradient and actually inhibit inflammation (Wahl, 1994). This may be a biofeedback mechanism that initially activates the inflammatory cascade locally and then, as TGF-ß escapes into circulation, negatively regulates it to prevent excessive inflammation. The TGF-ß1 (-/-) mouse model and the MRL/lpr mouse model are important examples of the consequences of excessive or too little host defense, and show that TGF-ß may provide the link between the processes by which tissues respond to insults and initiate repair. As TGF-ß is a critical determinant of host response to injury or infection, it is not suprising to find so many pathological conditions associated with its overproduction. Understanding the roles that TGF-ß may play in modulating the immune response may ultimately lead to more effective treatments for immune disorders.

b. Effects on Connective Tissues

TGF-ß is an important molecule in the biology of connective tissues. It stimulates fibroblast collagen, fibronectin, and GAG synthesis, enhances neovascularization, and modulates production of a variety of proteases and their inhibitors, ultimately resulting in the accumulation of matrix and scar production (Sporn et al., 1987; Allen et al., 1991; Overall et al., 1989; Edwards et al., 1987). TGF-ß upregulates type I collagen gene expression in normal fibroblasts (Ignotz et al., 1987). Similar effects have also been found in fibrotic disorders of the liver and in many fibroproliferative disorders, including scleroderma, myelofibrosis, hepatic, intraocular, and pulmonary fibrosis. Antagonism of TGF-ß effects with specific anti-TGF-ß antibodies blocked the progression of arthritis, glomerulonephritis, and pulmonary fibrosis, in animal models of these diseases. In normal wound healing, neutralizing antibodies to TGF-ß1 and TGF-ß2 lowered collagen content without compromising tensile strength, leading to more regenerative dermal reconstitution than fibrosis in dermal wounds in Sprague-Dawley rats (Shah et al., 1992). The architecture of the neodermis in the healing of dermal wounds more closely resembled the normal dermis, a situation recognized in fetal/embryonic wounds which also heal without scars but develop scars with provision of exogenous TGF-ß. Surprisingly, TGF-ß is present in higher amounts in fetal wounds than in adult healing wounds, which appears to contradict the previous findings (Longaker et al., 1994). It has been speculated that TGF-ß's biological activity may be reduced in fetal tissues due to the increased presence of TGF-ß neutralizing molecules. The differential regulation of the TGF-ß isoforms may also account for differences in wound healing. The concentration of the TGF-ß1 isoform in healing wounds is relatively constant regardless of the outcome, whereas TGF-ß2 concentrations are highest in wounds that heal without scarring (Longaker et al., 1994). Although the physiological importance of this observation is unclear, it does suggest that the isoforms have specific functions that may be important in the repair process.

These studies have identified an important role for TGF-ß in controlling cell proliferation and function *in vitro* and *in vivo* experimental wound healing systems. In normal repair processes, TGF-ß is highly regulated, forming associations with numerous inhibiting molecules that modulate its actions and

control its ability to induce its own production. A depressed ability to regulate it might lead to local or systemic overproduction of TGF-ß and may also account for many types of pathological repair processes that are characterized by excessive matrix deposition (reviewed by Border and Ruoslahti, 1992). Thus it is not suprising that a linkage has also been established between TGF-ß production and pathological wound healing.

C. THE INTERFERONS

Originally described for their actions as antiviral agents, the interferons are now recognized to be a multifunctional family of cytokines that are involved in many aspects of vertebrate biology (Burke and Isaacs, 1957). Two distinct classes of interferons have been observed based on their sequence identity and their interactions with cell surface receptors. Interferons α and ß (IFN-α and IFN-ß), termed type I interferons, are structurally and genetically related proteins that interact with a common receptor. The genes for the type I interferons are constitutively expressed at low levels by virtually all cell types, and undergo rapid and transient induction following viral infection (Pelligrini and Schindler, 1993). Interferon-γ (IFN-γ), a type II interferon, is produced only by T-lymphocytes and natural killer (NK) cells and interacts with a distinct cell surface receptor. While not related to the type I interferons, interferon-γ shares with them a common intracellular signal transduction protein (Sheehan and Schreiber, 1992). The multifunctional nature and biological importance of the interferon cytokine family has led to their use in the clinical management of numerous pathological conditions including fibrotic disorders.

Interferon Genes and Structural Features

a. Interferon-α

Twenty-four IFN-α genes code for structurally different human IFN-α proteins (Weissman and Weber, 1986). Eighteen of these genes, four of which are pseudogenes, code for IFN-αI proteins, while six genes, of which five are pseudogenes, code for IFN-αII proteins. There is higher sequence identity between the human and bovine IFN-αI genes than the human IFN-αI and -αII

genes, which has led some to estimate a divergence time for these two gene subfamilies of approximately 100 million years (De Maeyer and De Mayer Guignard, 1988). The human IFN-α genes have been localized to chromosome 9, where they occur in a tight cluster. They share similar structures and lack introns which suggests that they derive from a common ancestral gene. The 5' flanking regions of these genes contain a highly conserved 42 base pair purine-rich region immediately downstream from -117. They contain multiple GAA and GAAA sequences that are necessary for transcription (Fisher et al., 1983; Weidle and Weissmann, 1983). The 3' flanking regions of these genes are of variable length, and some have several polyadenylation sequences. They also contain ATTA and TTATTTAT repeats which occur on other cytokine genes and several protooncogenes, and are thought to confer instability to the transcripts (Caput et al., 1986). The IFN-α genes are inducible by pathogenic insult, and contain a regulatory sequence that is required for induction (Ryals et al., 1985). This sequence is located between positions -109 and -64 and contains two sets of repeats, a perfect pentameric repeat CAGAA, and an imperfect octomeric repeat A(A/T)GGAAAG.

The IFN-αI genes encode mature proteins of 165 or 166 amino acids, while the mature IFN-αII proteins are 172 amino acids long. The proteins are synthesized as pre-interferons, and contain a 23 amino acid leader sequence which is cleaved during maturation to yield the mature proteins (De Maeyer and De Maeyer-Guignard, 1988). They are characterized by four highly conserved cysteine residues which form two disulphide bridges from Cys1-99, and Cys29-139, the latter being essential for the cytokines' biological activity (Wetzel, 1981). The human proteins, unlike their murine counterparts, do not contain typical N-glycosylation sequences, and are generally not glycosylated (Petska et al., 1983).

b. Interferon-ß

Interferon-ß, commonly called "fibroblast interferon", differs from IFN-α in that it is the product of a single copy human gene also located on chromosome 9. The gene contains no introns and codes for a 166 amino acid mature protein (Derynck et al., 1980). It shares approximately 45% sequence identity with the IFN-α genes, while the proteins share approximately 30%

amino acid homology. The IFN-ß gene contains a promoter sequence called the inteferon gene regulatory element (IRE). The IRE is located between positions -77 and -37 and is required for maximal induction of the gene. It bears strong nucleotide sequence identity to the inducibility sequence of the IFN-α genes (Goodbourne et al., 1985).

IFN-ß is glycosylated and has an N-glycosylation site at position 180 (May and Sehgal, 1980). It also has three cysteine residues at positions 17, 31 and 141, with a disulfide bridge occuring between the latter two. When this bond is reduced, the biological activity of the molecule is lost (Mark et al., 1984).

c. Interferon-γ

Human IFN-γ is the product of a single copy gene located on chromosome 12. It is approximately 6 kb in length and contains four exons and three introns (Trent et al., 1982; Naylor et al., 1983). The introns are 1238, 295, and 32422 bp in length, while the exons code for 38, 23, 61 and 44 amino acids of the mature protein. The 5' flanking region of the human IFN-γ gene contains several regulatory sequences. A 200 bp region, which functions as a transcriptional enhancer, has high sequence identity to other T cell genes including IL-2 (Fujita et al., 1986). A second sequence located within the first intron also has 83% homology to the 5' flanking region of the IL-2 gene, though its functional significance is not known (Hardy et al., 1985).

The transcript is 1.2 kb and is translated into a 166 amino acid protein. Cleavage of the 23 amino acid signal sequence yields a 143 amino acid mature protein with a molecular weight of 17 kD (Derynck et al., 1982). The mature protein contains two N-glycosylation sites at positions 25 and 97. The independent and differential glycosylation of these sites gives rise to three biologically active forms of IFN-γ: a 17 kD unglycosylated form, a 20 kD form that is glycosylated at one site, and a 25 kD form that is glycosylated at both sites (Rinderknecht et al., 1984). Glycosylation does not appear to affect the function of the molecule; however, it influences the circulatory half-life. IFN-γ has a high content of basic residues which may explain its extreme sensitivity to acid (Devos et al., 1982). Incubation below pH 4.0 or higher than pH 9.0 causes rapid loss of its biological activity (Pace et al., 1983).

The biologically active form of IFN-γ is a homodimer of two polypeptides. The two molecules are not covalently associated, and little of the monomeric forms is detectable at physiological concentrations (Scahill et al., 1983). The crystallographic structure of IFN-γ resolved to 3.5Å, revealed that the molecule is 62% helical, and lacks ß-sheets. Each subunit consists of six α-helices held together by the non-helical regions (Ealick et al., 1991). Monoclonal antibodies against the amino termini of the cytokine abrogates its activity, while enzymatic digestion of the carboxy termini also impairs its function significantly (Johnson et al., 1982; Leinikki et al., 1987). Not suprisingly, the crystal structure of IFN-γ predicts that both the amino and carboxy termini of each monomer are exposed, suggesting involvement of these regions in receptor binding (Ealick et al., 1991).

Interferon Biosynthesis

Interferons are secreted by a number of cell types in response to specific stimuli. Induction of type I interferons is not a specialized cell function, as it is likely that most cells are able to activate these genes in response to viral infection or incubation with double-stranded RNA. Conversely, IFN-γ synthesis is induced by unique stimuli, and its production is limited to specifically sensitized NK cell and T-cell populations (Hauser, 1990; Sheehan and Schreiber, 1992).

a. Interferon α/ß

While different type I interferons share the same inducers, their production is not coordinately regulated, as certain cells can selectively produce specific interferons (reviewed by Hauser, 1990). Fibroblasts generally produce IFN-ß, while leukocytes produce IFN-α (Havell et al., 1978). The subtypes of IFN-α may also be expressed by specific cell populations (Hiscott et al., 1984).

The type I interferons can be induced by most animal viruses, regardless of their structure or mode of replication (reviewed by Ho, 1984). Other microorganisms are also able to induce their production including: rickettsia, bacteria, bacterial endotoxin, mycoplasma, a number of protozoa, and a variety of cytokines and growth factors (reviewed by De Maeyer and De Maeyer-Guignard, 1988).

The control if type I interferon biosynthesis occurs at both transcriptional and post-transcriptional levels. Structural changes in nucleosomes upstream of the type I interferon gene cluster have been observed preceding transcriptional induction (Bode et al., 1986). Furthermore, studies with the Sendai virus have shown that type I interferon gene transcription occurs rapidly in the presence of an inducer, and reaches a maximal level after which transcription terminates despite the continued presence of the inducer (Shuttleworth et al., 1983). Interestingly, pre-treatment of cells with IFN-ß prior to addition of an inducer causes a ten-fold increase in the transcription of the gene, suggesting autocrine control at some level (Nir et al., 1985). The posttranscriptional control of type I interferon biosynthesis is thought to occur at the level of mRNA stability and during translation. Messenger RNAs for IFN-α/ß are highly unstable and subject to rapidly degradation (Caput et al., 1986). Increasing the half-life of these transcripts, in some instances, results in increased protein production, though in certain cell lines the presence of mRNA for IFN-α is detectable in the absence of active IFN-α synthesis (Morser and Shuttleworth, 1981; Berger et al., 1980).

b. Interferon-γ

T-lymphocytes are the major source of human IFN-γ. Both CD-8$^+$ and certain subsets of CD4$^+$ cells are able to produce the cytokine, and its production has been associated with the selection of specific immune response pathways. Using murine T-cell clones *in vitro*, Mossman and Coffman (1989) showed that different subsets of T helper populations can be distinguished by their cytokine production patterns. They determined that T_H1 lymphocytes preferentially produce IL-2, IFN-γ, and TNF-α, and select for cell mediated immunity while inhibiting the progression of humoral immune pathways. Conversely, T_H2 cells were found to produce high amounts of IL-4, IL-5, IL-6 and IL-10. They further characterized a T_H0 phenotype of cells capable of producing all types of cytokines. This model has provided a convenient framework for understanding the mechanisms controlling of the immune system and its ability to respond to microbial pathogens.

The characterization of IFN-γ as a T_H1 cytokine then, suggests that its biosynthesis may be controlled at the level of immune class selection. Several stimuli have been shown to activate IFN-γ expression *in vitro*: antibody

stimulation of the T-cell receptor/CD-3 complex, T-cell mitogens, calcium ionophores, IL-2, leukotrienes, and hydrogen peroxide (reviewed by Sheehan and Schreiber, 1992). Induction of IFN-γ transcription occurs immediately upon T-cell stimulation, peaks between 12 and 24 hours and then falls quickly (Schreiber et al., 1983).

Natural killer cells produce IFN-γ in response to bacterial or microbial stimulation (Bancroft et al., 1987). In a *scid* mouse model, IFN-γ from NK cells can provide their immunodeficient host with partial resistance to infection with *Listeria monocytogenes*. Interferon-γ production by NK populations may, therefore, represent the host's first line of defence against pathogenic insults (Wherry et al., 1991).

Interferon Receptors

It is now well established that the Type I interferons cross-react with a common receptor, while IFN-γ binds a distinct cell surface receptor (reviewed by Pelligrini and Schindler, 1993; Uzé et al., 1995). While the exact mechanisms coupling receptor binding to signal transduction have not been well ellucidated, a number of recent studies have provided insight into the structure and function of the IFN receptors.

a. Interferon-α/ß Receptor

The nature and identity of the IFN-α/ß receptor has been a difficult area to resolve, and only recently has some degree of understanding been reached (Uzé et al., 1995). Several genetic studies have revealed that the sensitivity of cells to IFN-α/ß and -γ is related to the presence of loci located on human chromosome 21 (Slate et al., 1981). The IFN-α/ß receptor gene (*ifnar1*) has subsequently been localized to 21q22.2-3, and codes for a component of the IFN-α/ß receptor complex (IFNAR). The predicted structure of the extracellular domain of IFNAR identifies it as a class II member of the cytokine receptor family (Uzé et al., 1995). The cross-linking of radioiodinated IFN-α to isolated cell membranes revealed 110-150 kD bands, which has suggested the 100-130 kD high affinity IFNAR may be physically associated to other proteins, including the protein tyrosine kinase tyk2 (Calamonici and Domanski, 1994). The molecular mass of this receptor component seems to

vary with cell origin. Furthermore, it appears to be present in rather low abundance on cell surfaces (100-5000 molecules per cell) making isolation and purification extremely difficult (Novick et al., 1994).

It is now becoming clear that the IFN-α/ß receptor is in fact a multimeric heterogeneous complex. Several other components of the complex have recently been identified. A 65 kD antigenically distinct IFN binding protein that does not coimmunoprecipitate with the IFNAR, and another distinct 105 kD glycoprotein that does co-immunoprecipate, have been isolated (Constantinescu et al., 1994; Eid and Tovey, 1995). Interestingly, a soluble 45 kD IFN-α/ß receptor, dimerically bound to an unidentified protein, has been isolated from human urine, and has recently been cloned and sequenced (Novick et al., 1994). It is tentatively thought to be the same protein as the 65 kD cell surface glycoprotein isolated by Constantinescu et al. (1994). Thus, the number of components comprising the IFN-α/ß receptor, and their specific functions have not yet been determined, though it appears that the receptor is in fact a multi-domain protein complex that may display cell specific heterogeneity.

b. Interferon-γ Receptor

Interferon-γ displays high affinity binding to a ubiquitously expressed glycoprotein cell surface receptor (IFN-γR). Immunochemical, radioligand binding, and genetic analyses have demonstrated that the IFN-γR is expressed on all somatic and hematopoetic cell types, excluding erythrocytes (Sheehan and Schreiber, 1992). Upon capture, the receptor/ligand complex is internalized into the endosomal compartment. The free receptor recirculates while the ligand is transported to the lysosome for degradation.

The IFN-γR, encoded on chromosome 6, is sufficient to bind the cytokine when expressed on cell surfaces (Aguet et al., 1988). It is unable to confer biological activity on IFN-γ unless complexed to a species-specific transmembrane glycoprotein encoded on chromosome 21, which does not contribute to the extracellular binding of IFN-γ (Soh et al., 1994). The IFN-γR gene is approximately 30 kb, and contains six exons which result in a 2.3 kb transcript. The gene product is an 472 amino acid protein, which, when glycosylated, gives an 80 kD single-chain glycoprotein receptor (Hershey et al., 1989). It is symetrically oriented around a 23 amino acid transmembrane

domain, and has a 228 amino acid extracellular domain containing ten cysteine residues and five occupied N-glycosylation sites (Sheehan and Schreiber, 1992). The intracellular domain of the IFN-γR is long and contains multiple serine and threonine residues. Upon stimulation of the receptor, specific serine and threonine residues become phosphorylated (Khurana Hershey et al., 1990). A 48 amino acid juxtamembrane region and a three amino acid (Tyr-Asp-His) carboxy-terminal portion have been identified as being critical for function. Furthermore, residues 434-472 are necessary for IFN-γ-stimulated MHC class I expression in human fibroblasts (Farrar et al., 1991).

Signal Transduction

The downstream consequences of IFN receptor stimulation have been well described in recent years. While both the type I interferons and IFN-γ bind different receptors and elicit different biological effects from target cells, there is some redundancy in the intracellular cascade they effect.

Neither the IFNAR nor any of the accessory IFN-α/ß binding molecules in the receptor complex contain a functional enzyme that becomes activated upon ligand binding. Instead, they recruit cytoplasmic tyrosine kinases from the Janus family, known as tyk2 and jak1 (reviewed by Uzé et al., 1995; Pelligrini and Schindler, 1993; Darnell Jr. et al., 1994). These kinases phosphorylate latent cytoplasmic proteins known as STATs (signal transducers and activators of transcription). In the IFN-α/ß pathway, two proteins are known to be activated by phosphorylation: STAT1 (p91), and STAT2 (p113). When phosphorylated on Tyr701 (STAT1 by jak1 and STAT2 by tyk2), these proteins combine and translocate to the nucleus, where they bind a third protein, p48 DNA binding protein, to form the transcription factor ISGF-3 (interferon-stimulated gene factor-3). ISGF-3, by virtue of its DNA binding ability, binds and transactivates target genes through specific promoter sequences termed interferon-stimulated response elements .

Interferon-γ-stimulated signal transduction occurs by a similar mechanism. The binding of IFN-γ to its receptor causes the recruitment of jak1, and the subsequent phosphorylation of STAT1 at Tyr701. STAT1 is thought to translocate to the nucleus where it oligomerizes to form gamma-interferon activated fragment or GAF. GAF then binds and transactivates genes

containing the gamma-interferon activated sequences, or GAS sequences. It is not known whether STAT1 is the only protein required for Interferon-γ-stimulated gene upregulation, or even if it is the only member of GAF, though antibodies to the other known STAT proteins do not bind GAF.

Anti-fibrogenic Effects of Interferons

A number of *in vitro* studies have demonstrated that the interferon family has profound effects on mesenchymal cell metabolism and consequently may be important regulators of wound healing. IFN-α, -ß, and -γ are able to reduce the synthesis of collagen and inhibit the proliferation of human fibroblasts (De Maeyer and De Maeyer-Guignard, 1988). The mechanisms by which collagen synthesis is downregulated are unclear, though the observation that transcription of the types I and III procollagen genes is unaffected by treatment may suggest that regulation occurs at the level of transcript stability (Czaja et al., 1989). Harrop et al. (1995) demonstrated that IFN-γ reduces collagen mRNA expression and has antiproliferative effects on normal and hypertrophic scar fibroblasts within 12 hours of incubation. In the same study, IFN-α2b elicited the same effects, though after 72 hours incubation. Once effective, IFN-α2b reduced newly synthesized collagen at a number of different levels: decreasing type I collagen mRNA production, and stimulating the intracellular degradation of newly synthesized collagen (Tredget et al., 1992). Northern blot analysis has demonstrated a significant upregulation of TIMP I and collagenase mRNA levels following treatment with IFN-α2b, further demonstrating its anti-fibrogenic nature (Ghahary et al., 1993).

Clinical Applications of Interferons in Fibrosis

Both IFN-α and -γ have been used extensively in the treatment of disease. Their uses in the management of fibrotic diseases have occurred relatively recently, though there is now ample evidence to suggest that the interferon family can regulate the progression of fibrosis in animals and humans.

Using murine wound healing models, Granstein et al. (1990) showed the efficacy of IFN-γ treatment at inhibiting fibrosis and collagen deposition in argon laser-induced full thickness wounds. Futhermore, in a murine

schistosomiasis liver fibrosis model, reductions in type I procollagen mRNA production were found following IFN-γ treatment (Czaja et al., 1989).

In human diseases, subcutaneously administered IFN-α has been employed for the treatment of numerous diseases including hepatitis C, while IFN-γ therapy has been used for the treatment of hypertrophic scars, rheumatoid arthritis, and membranous glomerulonephritis (Pittet et al., 1994; Veys et al., 1988; Jonas et al., 1991). Patients suffering from chronic hepatitis and cirrhosis responded well to IFN-α treatment, wherein six of eight patients treated demonstrated normalization of serum procollagen type III peptide, liver enzyme function, and TGF-ß1 mRNA in liver tissues at the end of one year (Castilla et al., 1991).

Deficiencies in the endogenous production of interferons are thought to lead to succeptibility to fibrosis. Vicente-Gutierrez et al. (1991) proposed that a deficiency of endogenous IFN-α and -γ production may be a contributing factor to liver fibrosis following toxic damage. An endogenous deficiency in production of these same molecules has also been suggested as causative in the development of keloids scars (McCauley et al., 1992).

Thus, the anti-fibrogenic effects of the interferons both *in vitro* and *in vivo* and their implication in the development of fibosis, suggests that they are important regulators of wound healing and are useful agents in the clinical management of fibrotic disorders.

D. CONCLUSION

The complexity of the interplay between cells and macromolecules in wound healing is astonishing. Inititation of specific signaling pathways, the accessibility of cytokines and growth factors to their receptors, and the numerous and often paradoxical cellular effects that they elicit, depend on an infinite variety of conditions. While an enormous amount of information is currently available concerning the individual functions of cytokines in controlled environments, there is very little understanding of their roles *in vivo*. Resolution of the true nature of these intricate systems will ultimately result in an understanding of one of evolution's most important homeostatic processes.

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CHAPTER 3 - ESTABLISHMENT OF A SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE QUANTITATION OF TGF-ß1 IN HUMAN SERUM

INTRODUCTION

The identification of TGF-ß as an important biological molecule has necessitated the development of specific quantitative assays for both its accurate measurement and determination of its biological activity. A number of different assay systems are currently used to detect TGF-ß, though its multifunctional nature, the redundancy in the actions of its isoforms, and its poor antigenicity due to high structural conservation, have led to numerous problems with accuracy and reproducibility (reviewed by Meager, 1991). The difficulties involved in the measurement of TGF-ß are a consequence of its unique biochemical features. It is synthesized in a precursor latent form which does not bind receptors or antibodies, and has no biological activity (reviewed by Roberts et al., 1990). Furthermore, evidence of post-transcriptional regulation of TGF-ß suggests that examination of mRNA levels of the molecule may not sufficiently reflect protein production (Coletta et al., 1990; Kim et al., 1992).

Activation of the latent complex, by proteolysis or dissociation of latency proteins, confers biological activity upon the molecule and allows it to interact with other macromolecules including antibodies, receptors and certain TGF-ß binding proteins, α2-macroglobulin among them (O'Conner-McCourt and Wakefield, 1987). Currently used activation procedures depend on the source of TGF-ß. Total circulating TGF-ß (primarily TGF-ß1) obtained from serum or plasma can be extracted and partially purified by an acid-ethanol differential extraction protocol (Roberts et al., 1980).

Assays for TGF-ß fall into two categories: biological and immunological. The biological assay systems compare the cell-type specific effects of different concentrations of purified TGF-ß to those generated from unknown samples. The immunological assays involve the use of purified monoclonal and polyclonal antibodies, as well as recombinant purified TGF-ß receptors for the estimation of known and unknown concentrations of the cytokine by ligand capture and detection. The biological assays are generally used to detect biological activity, and are of little use as quantitative tools. While the

immunoassays are highly specific, they may not be useful in the determination of TGF-ß biological activity. Both types of assays are extremely sensitive, though the specificities and the utility of the information generated from each assay system are different and must be considered carefully.

Biological Assay Systems

The biological assays for TGF-ß are numerous and each relies on unique aspects of the growth factor's many functions. Three major biological properties of TGF-ß are commonly used in biological assay systems: its ability to inhibit the growth of cells in culture, its ability to stimulate the anchorage independent growth of cells, and its ability to upregulate the expression of specific genes in target cells.

TGF-ß has been shown to be growth inhibitory for a variety of cell types, including epithelial, endothelial and lymphoid-derived cells (Meager, 1991). For bioassays, the mink lung epithelial cell line Mv-1-Lu (or CCL-64) is the most widely used, and is strongly growth-inhibited by relatively low concentrations of TGF-ß (Tucker et al., 1984; Like and Massagué, 1986). The loss of proliferative capacity in cultured CCL-64 cells is estimated by determining the [^{3}H]thymidine incorporation into the DNA of cells incubated with multiple known and unknown concentrations of TGF-ß. Growth inhibition by unknown samples is compared to a standard curve generated from the inhibitory effects of known concentrations of purified TGF-ß, and the concentrations of the unknown samples are then interpolated. The sensitivity range of the assay has been reported to be 0.08-10 pM. Growth inhibitory assays have been done with a number of different cell lines, including the MOSER human colon carcinoma cell line, the A375 human melanoma cell line, and the TF-1 human erythroleukaemia cell line (Hoosein et al., 1987; Brown et al., 1987; Randall et al., 1993). Though these assays are widely used, their relative sensitivities and specificities are problematic. The sensitivities of individual strains of the same cell line are different, and in some cases, cells lose their sensitivity to TGF-ß altogether (Meager, 1991). Several research groups have generated CCl-64 cells that become resistant to TGF-ß after numerous passages and prolonged treatment with different concentrations of the cytokine (Boyd and Massagué, 1989; Laiho et al., 1990). The major problem with growth inhibition assays, however, is their lack of specificity. Numerous

other cytokines and growth factors have been shown to stimulate or inhibit the growth of cells used in TGF-ß bioassays, including TNF-α/ß, IL-1α/ß, and IFN-γ (Brown et al., 1987; DeBenedetti, 1990). Furthermore, TGF-ß-binding proteins in experimental samples, such as the serum protein α2-macroglobulin, have been shown to inhibit the biological activity of TGF-ß (Danielpour and Sporn, 1990). Lastly, due to the similarity in function of the TGF-ß isoforms, specifically TGF-ß1, TGF-ß2, and TGF-ß3, bioassays are not able to distinguish among their activities (Meager, 1991). A variety of factors, therefore, compromise the validity of growth inhibitory bioassays for TGF-ß.

TGF-ß was termed a transforming growth factor for the observation that it was capable of inducing the reversible anchorage-independent growth of certain murine cell lines in the presence of other growth factors (Moses et al., 1981; Roberts et al., 1980). The NRK 49-F assay, developed by Roberts et al. (1981), was the first method used to quantify the biological activity of TGF-ß. Normal rat kidney fibroblasts of the NRK 49-F cell line, when suspended in semi-solid agar containing bovine calf serum, TGF-ß, and a fixed concentration of either TGF-α or EGF, proliferate to form colonies within 7-10 days. Comparison of the size of the colonies incubated with known and unknown amounts of TGF-ß over a fixed time interval, is then used to extrapolate the amount of TGF-ß present in unknown samples. Several other cell lines have been used in this assay system, including the AKR-2B mouse cell line, the BALB/c 3T3 cell line, and the A431 human epidermoid carcinoma cell line (Moses et al., 1981; Massagué et al., 1985; Rizzino et al., 1986). The problems associated with this technique are so numerous that it is rarely used as a quanitative method (Meager, 1991). A number of growth factors other that TGF-ß can induce or potentiate the transformed phenotype of these cell lines. PDGF and FGF augment NRK 49-F colony formation on soft agar. As PDGF is found in variable amounts in fetal calf serum, it is a potential source of artifact in experimental conditions (Jullien et al., 1989). Furthermore, as with the growth inhibition assay systems, cell lines that have lost sensitivity to TGF-ß or display TGF-ß-independent soft agar growth have been isolated (Nugent et al., 1989). The protocol for this technique is also extremely time consuming, and the measurements and comparisons of colony sizes are highly qualitative and error prone (Randall et al., 1993).

A third type of biological assay used for the detection and quantitation of TGF-ß relies on its ability to induce the expression of specific genes. One such

assay focuses on the upregulation of the plasminogen activator inhibitor-1 (PAI-1) gene. A number of groups have demonstrated the ability of TGF-ß to upregulate specifically the gene for PAI-1 *in vitro* (Keeton et al., 1991; Wrana et al., 1992). Abe et al. (1994) used this observation to develop a sensitive bioassay for TGF-ß. An expression vector containing a truncated PAI-1 promoter fused to the firefly luciferase gene was stably transfected into mink lung epithelial cells. TGF-ß added in concentrations between 0.2 and 30 pM caused a dose-dependent increase in the luciferase activity of cell lysates, while other inducers of PAI-1 such as bFGF, PDGF-BB, and EGF were required in doses greater than 500 pM. The addition of plasmin and thrombin in this system, however, induced expression of the construct, and samples required neutralization with aprotinin. Though this is a highly sensitive bioassay (0.2pM-30 pM), it succumbs to the same essential pitfalls of all biological assays: it is not isoform specific, it may not be TGF-ß specific, and a large number of variables remain uncontrolled. Furthermore, as this is an indirect assay relying on the production of the luciferase protein for detection of TGF-ß activity, luciferase mRNA expression and trafficking must not be affected by the addition of pharmacological agents.

Immunological Assay Systems

The lack of specificity and reproducibility of biological assay systems for TGF-ß preclude their use as quantitative tools. The development of immunoassays has to some degree circumvented this problem, as these systems are sensitive, reproducible, and easily performed (Meager, 1991). Two types of immunological assays have been developed: antibody-based sandwich enzyme-linked immunosorbent assays (SELISAs), and receptor binding assays.

The development of antibodies specific for TGF-ß has proved rather difficult as it is both a conserved, and immunosuppressive molecule. Antibodies that were produced until recently were in limited supply and not commercially available (Meager, 1991). Immunization of rabbits with porcine TGF-ß1 resulted in antibodies being produced from three of twelve animals (Keski-Oja et al., 1987). The antibodies, however, were crossreactive with the TGF-ß2 isoform and hence not specific. Danielpour et al. (1989a), were able to generate turkey antibodies specific for both TGF-ß1 and TGF-ß2 isoforms but

few animals were able to produce them. Subsequently, the same group developed a sensitive and specific ELISA based on both rabbit and turkey antibodies (Danielpour et al., 1989b). Mouse monoclonal antibodies against both human and bovine TGF-ß isoforms have also been developed and have proved extremely useful in the development of immunological assays (Lucas et al., 1990; Dasch et al., 1989). The recent availability of a variety of commercial anti-TGF-ß antibodies has led to the development and refinement of a reproducible, sensitive, and isoform specific SELISA (Danielpour, 1993). The basis of the assay is antibody-antigen interaction followed by colorimetric detection. Concentrations of TGF-ß are related to spectrophotometric optical density determinations to generate a standard curve from which unknown TGF-ß concentrations can be extrapolated. While less sensitive than most biological assays (20-2000 pg/ml), this system provides higher specificity, easier reproducibility, and quantitative data. The information that it provides concerning the biological activity of TGF-ß is limited. Latent TGF-ß does not interact with antibodies or receptors prior to activation (Wakefield et al, 1988). Any TGF-ß bound to antibody, then, can be considered biologically active, though the regulation of TGF-ß activity by other factors cannot be determined by antibody detection (Meager, 1991).

A different technique used for the measurement of biologically active TGF-ß is a receptor-binding assay. Two types of TGF-ß receptor-binding assays are commonly used: radio-receptor assays, and receptor based SELISAs. Radio-receptor assays are useful quantitative tools if it is known that only one subtype of TGF-ß is present in an unknown sample. Contamination of experimental samples with other TGF-ß isoforms can inhibit the binding of a specific isoform (Meager, 1991). Receptor-ligand interactions are the basis of these assays. Human A549 lung carcinoma cells, known to contain TGF-ß receptors, are cultured and briefly incubated different concentrations of ^{125}I-TGF-ß and unknown concentrations of non-radioactive TGF-ß. Competition from the unknown samples prevents the binding of the iodinated cytokine to the surface receptors at different. A competition standard curve is generated from gamma counts of the cultured cells, and concentrations of the unknown samples can be extrapolated (Arrick et al., 1990). These assays specifically reflect receptor-binding, and hence provide information regarding the biological activity of specific TGF-ß isoforms. They are, however, extremely time consuming and require highly

specialized conditions for accurate results. Receptor-based SELISAs are almost identical to antibody-based SELISAs, the only difference being the substitution of the primary monoclonal antibody with a recombinant TGF-ß receptor (Grainger et al., 1995). The TGF-ß type II receptor, primarily responsible for the extracellular capture of the cytokine on cell surfaces, was expressed in *E. coli* as a truncated GST-fusion construct, purified, and conjugated to microtiter wells in a SELISA. The advantage to this technique may be that it reflects more accurately the actions of TGF-ß *in vivo*, and can be used to gauge biological activity.

In this thesis, a SELISA for the specific detection of the human TGF-$ß_1$ isoform is established based on the methodology of Danielpour (1993). The parameters of the assay are determined, and it is used to quantify TGF-ß levels present in acid-ethanol extracts of human serum.

MATERIALS AND METHODS

Materials

Ultrapure natural human TGF-$ß_1$ and mouse monoclonal anti-TGF-$ß_{1,2,3}$ IgG1 were purchased from Genzyme (Cambridge, MA, cat. no. 1835-01, and 1294-01). Immulon I microtiter plates and wells were purchased from Fischer Scientific (Ottawa, ON). Crystalline bovine serum albumin (BSA), alkaline phosphatase-conjugated rabbit anti-chicken IgG, and alkaline phosphatase substrate (p-nitrophenylphosphate disodium) were obtained from Sigma chemical co. (St. Louis, MO, cat. no. A-4378, A-9171, and P-104). Chicken anti-TGF-ß neutralizing antibody was obtained from R&D Systems, Inc. (Minneapolis, MN, cat. no. AB-101-NA).

Human Serum

Human serum samples were generously provided by the Canadian Red Cross. Whole blood from twenty-six healthy male donors between the ages of 19 and 66 years, was taken in collection vials without the presence of an anticoagulant. The samples were allowed to clot, and then centrifuged at 4°C and 3,000 rpm for 30 minutes. The supernatant was then aliquotted into 1.5 ml Eppendorf tubes and stored at -80°C until use.

Acid-Ethanol Extraction of TGF-ß from Serum

Total TGF-ß (both active and latent) was extracted from serum as described by Roberts et al. (1981). Briefly, 0.5 ml serum samples were diluted 1:1 with distilled water, vortexed, and then acidified with 4.0 ml of acid-ethanol solution (93% ethanol, 2% concentrated HCl (0.24M), 85 mg/ml PMSF, 5mg/ml Pepstatin A). The samples were then vertically rotated overnight at 4°C, and centrifuged at 10,000Xg for 30 minutes. The supernatants were collected and stored at 4°C, and the pellets were re-extracted by another overnight vertical rotation at 4°C in 4.0 ml of acid-ethanol solution. After re-centrifugation at 10,000 X g for 30 minutes, the pellets were discarded, the two supernatants were then combined and adjusted to pH 5.2 with 12M ammonium hydroxide. The samples were buffered with 2M ammonium acetate pH 5.3 (1.0 ml for every 85 ml of supernatant), and diluted 1:3 with cold 99% ethanol (-20°C). The proteins were precipitated at -20°C for 48 hours, and then centrifuged at 10,000 X g for 30 minutes. The supernatants were discarded, the pellets dissolved in 4.0 ml of 1M acetic acid, and dialyzed against 1% acetic acid in Spectropore dialysis tubing (6000-8000 M.W.-cutoff). The samples were then frozen at -20°C, and lyophilized to yield a partially purified TGF-ß product.

SELISA for TGF-ß1

The TGF-ß SELISA was modified from the methodology of Danielpour (1993). Immulon I microtiter wells were coated with 100 µl/well of a mouse monoclonal anti-TGF-$ß_{1,2,3}$ primary antibody (1mg/ml) in Dulbecco's phosphate buffered saline (PBS), incubated at room temperature for 60 minutes, and then overnight at 4°C. The wells were then warmed to room temperature for 20 minutes, the antibody solution was discarded, and the wells washed once (200 µl/well) with a PBS/0.05% Tween 20 wash solution (WB). The wells were then blocked for 60 minutes(175 µl/well) with 1% crystalline BSA solution in Tris buffered saline (150 mM NaCl, 100 mM Tris, pH 7.6). Stock ultrapure human TGF-$ß_1$ in sterile PBS (20 mg/ml) was then diluted 1:160 in BB1 assay buffer (0.1% crystalline BSA, 0.1% Tween 20, 0.02% sodium azide, 150 mM NaCl, 100 mM Tris, pH 7.6) to give a diluted stock

concentration of 0.125 ng/ml. The diluted stock concentration was further diluted in BB1 to give standard concentrations for the assay (0-250 pg/100ml). Lyophilized experimental samples were also diluted (1:720) in BB1 assay buffer, and then both experimental and standard TGF-ß samples were incubated in triplicate in ELISA wells (100 µl/well) for 60 minutes. Following 3 washes (200 µl/well) with WB (5 minutes each), the wells were incubated (100 µl/well) with a 1:200 dilution of the stock concentration of chicken-anti-TGF-ß polyclonal antibody (1 mg/ml) for 60 minutes. The wells were then washed 5 times (200 µl/well) with WB (5 minutes each), and incubated for 60 minutes (100 µl/well) with a 1:3000 dilution of the stock concentration of rabbit-anti-chicken IgG alkaline phosphatase. After 5 more washes (200 µl/well) with WB (5 minutes each), the wells were incubated (100 µl/well) with a 1 mg/ml concentration of p-nitrophenylphosphate in 1.0 M diethanolamine buffer (pH 9.8). Colour was allowed to develop for 1-1.5 hours at room temperature, and then the wells were read on an automated spectrophotometric plate reader (EAR400AT, SLT Instruments, Austria) at 405 nm. For quantitation of unknown concentrations of TGF-ß$_1$, triplicate data points were analyzed by regression analysis and interpolation of the standard curve.

RESULTS

Standard Curve

In order to quantify effectively TGF-ß levels in experimental samples, a standard curve must be established with known concentrations of TGF-ß, and the appropriate development and assay conditions must be established.

To determine the conditions necessary to generate a linear relationship between serial concentrations of TGF-ß and spectrophotometric optical density (O.D.) readings, dilutions of human ultrapure TGF-ß$_1$ in BB1 assay buffer (0-250 pg/100ml) were used in a SELISA. A linear relationship was observed when developed for 1 hour. When the ELISA was developed for longer than 2 hours, the relationship among the data points was no longer linear, and displayed saturation kinetics (Figure 3-1). Linear standard curves with 1 hour development times, were therefore generated, with resulting correlation coefficients >0.95 (Figure 3-2).

The sensitivity of the assay system was determined by adding two standard deviations to the mean optical densities of 10 zero standard replicates and calculating the corresponding concentrations from the standard curve. The sensitivity limit was calculated to be 15 pg/100ul (data not shown).

Measurement of TGF-ß1 levels in Human Serum

TGF-ß1 was partially purified from the serum of 26 healthy male blood donors by acid-ethanol extraction, and the lyophilized partially purified protein was diluted 1:720 in BB1 buffer and analyzed by SELISA, yielding data points that fell within the linear portion of the standard curve (Table 3-1). The population had a mean serum TGF-ß1 level of 56.85±8.38 ng/ml. Serum TGF-ß1 concentrations ranged from 43.64±0.73 ng/ml to 74.31±4.63 ng/ml. There was no obvious age-related trend in serum levels.

Establishment of an assay system requires the determination of its variability. The overall variability in the assay system was determined by repetitive extraction and measurement (n=10) of a serum sample pooled from the donor population. The coefficient of variation in the measurements was calculated to be 0.231.

DISCUSSION

The immunological assay system reported by Danielpour (1993) is more sensitive than that described here. The sensitivity of this SELISA was found to be 14.9 pg/100ml compared to 2.5 pg/100ml previously reported. The linear range of this assay was, however, sufficient for the reproducible determination of TGF-ß1 from human serum. The range of the normal male population measured was small and tightly distributed around the population mean. Though the inter-assay variability is calculated to be 0.231, it includes all possible sources of error: variability from the collection and storage of the serum, from repeated acid/ethanol extractions, from repeated SELISAs, and from any other sources.

Table 3-1.

Serum TGF-ß1 levels in male blood donors

Number	Age (years)	Serum TGF-ß (ng/ml) (mean±S.D.)
M1	64	48.50±0.89
M2	56	61.07±9.91
M3	43	59.33±4.89
M4	31	68.89±5.41
M5	42	71.38±7.95
M6	51	74.31±5.67
M7	44	43.63±0.89
M8	41	50.79±10.09
M9	43	64.11±7.81
M10	39	55.16±2.42
M11	37	51.08±1.66
M12	34	44.74±5.11
M13	33	51.93±6.17
M14	40	53.79±6.85
M15	19	59.84±1.94
M16	53	45.12±6.60
M17	20	54.42±6.26
M18	66	54.91±2.60
M19	29	56.83±5.24
M20	27	44.36±3.63
M21	32	63.10±4.28
M22	70	53.49±4.36
M23	48	53.36±2.36
M24	36	63.56±9.42
M25	30	54.21±5.83
M26	22	54.42±6.26
mean	40.4	56.85±8.38

Table 3-1. Serum levels of TGF-ß1 were interpolated from a standard curve for a population of male blood donors (M1-26). No age related fluctuations were observed.

Figure 3-1.

Saturation kinetics of serial TGF-ß1 dilutions

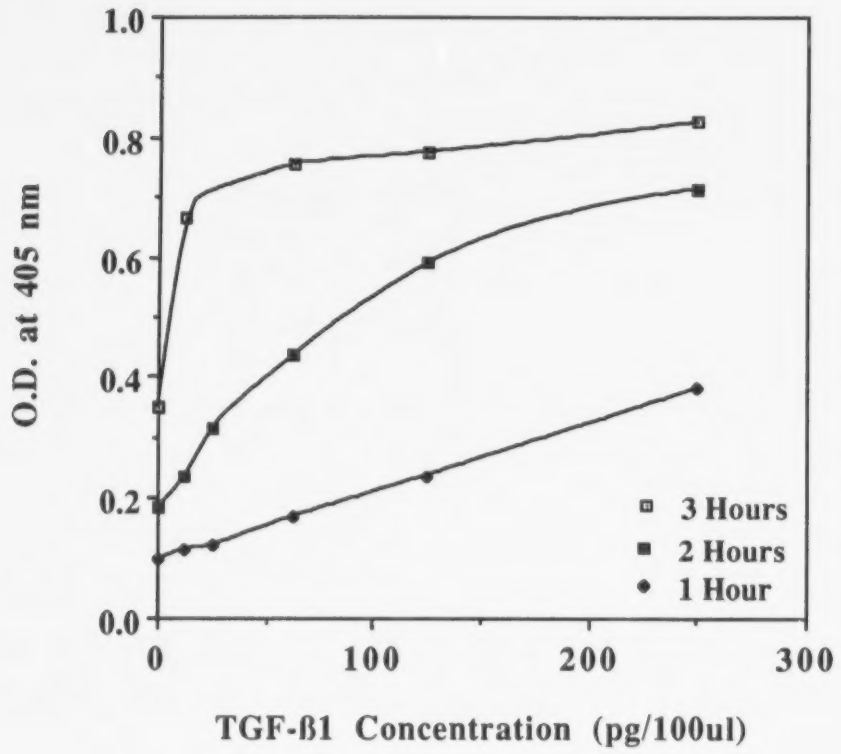

Figure 3-1. Serial dilutions of purified human TGF-ß1 display saturation kinetics when developed for longer than 2 hours.

Figure 3-2.

Regressed linear standard curve for TGF-ß1

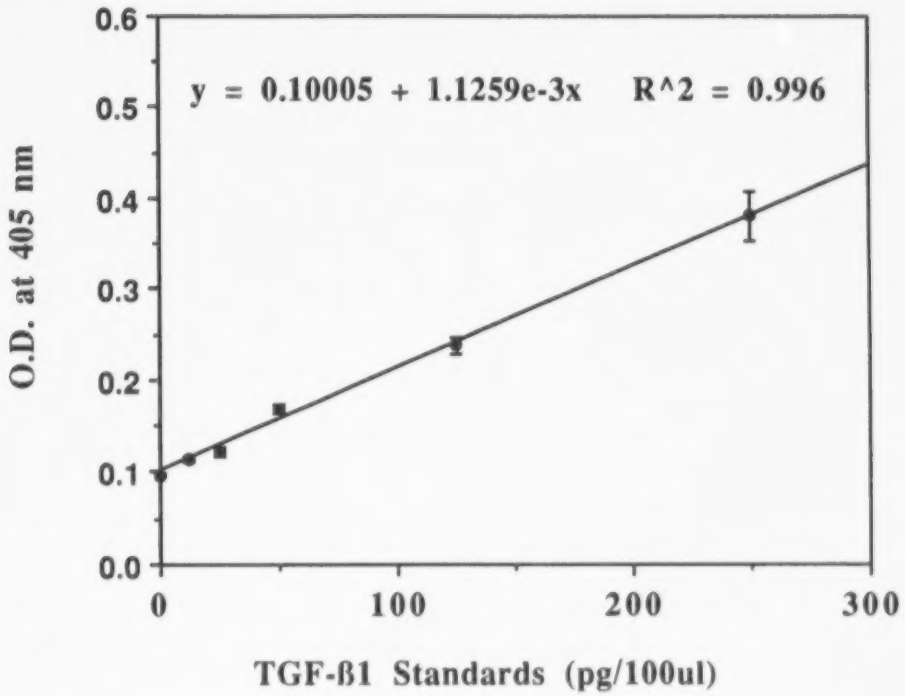

Figure 3-2. Standard curves for TGF-ß1 developed for 1 hour are linear and can be regressed with correlation coefficients >0.95.

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CHAPTER 4 - THE ROLE OF SYSTEMIC TGF-ß IN HYPERTROPHIC SCARRING POST-BURN: ANTAGONISM BY IFN-α2b

INTRODUCTION

Fibroproliferative disorders are common and often serious pathological conditions that can involve a variety of human tissues. Common examples of these disorders include cirrhosis and fibrosis of the liver, pulmonary fibrosis, myelofibrosis, rheumatoid arthritis, and scleroderma (Rockwell et al., 1989). Hypertrophic scarring (HSc) is a fibroproliferative disorder of the human dermis that often complicates the healing of deep second and third degree burns. These scars pose serious cosmetic problems and can cause functional impairment and significant morbidity in afflicted individuals due to the frequent presence of contractures (Scott et al., 1994). Hypertrophic scars are characterized by the formation of raised, erythematous, and pruritic scar tissue due to excessive and disorganized deposition of extracellular matrix components, primarily collagens, during the healing process (Linares et al., 1972). The clinical management of HSc includes pressure garment therapy, local injection of corticosteroids, and in severe cases surgical revision. While providing some relief to afflicted individuals, these therapies are slow, labor intensive, and only partially effective (Scott et al., 1994).

While the pathogenesis of HSc formation is not well established, it appears to be related in part to excessive extracellular matrix depostion by wound fibroblasts due to the persistence of fibrogenic growth factors in the wound environment. Immunohistochemical and northern blot analyses comparing normal and HSc tissue sections from the same patient, demonstrated an increased presence within HSc sections of the fibrogenic cytokine TGF-ß (Scott et al., 1995; Ghahary et al., 1993).

TGF-ß is a multifunctional cytokine that is ubiquitously expressed by virtually all cell types. Among its many functions, TGF-ß is highly fibrogenic and is an important cytokine in the wound healing cascade (Roberts et al., 1986). In an animal model of experimental glomerulonephritis, Border et al. (1992), demonstrated that neutralization of TGF-ß by systemic administration of antibodies or the proteoglycan decorin supressed glomerular extracellular matrix production. Furthermore, antibodies to TGF-ß have been shown to

control scar tissue formation in rat incisional wounds (Shah et al., 1992). Overproduction of TGF-ß has also been correlated to the development of a number of human fibroproliferative disorders, including glomerulonephritis, hepatic and pulmonary fibrosis, and radiation pneumonitis (Yoshioka et al., 1993; Castilla et al., 1991; Anscher et al., 1994). Recently, high plasma levels of TGF-ß have been shown to be predictive of liver and lung fibrosis in patients who received autologous bone marrow transplantation for advanced breast cancer (Anscher et al., 1993). These studies suggest that local and systemic TGF-ß can influence wound healing and may play a role the etiology of fibrosis.

The present studies compare serum TGF-ß levels of patients with post-burn HSc to serum levels in a population of sex matched healthy individuals. Levels of TGF-ß are also examined in the same patient population over the course of their treatment with systemic IFN-α2b.

MATERIALS AND METHODS

Materials

Human recombinant Intron A^R used in the clinical trial was obtained from Schering (Pointe Claire, Quebec). Ultrapure natural human TGF-$ß_1$ and mouse monoclonal anti-TGF-$ß_{1,2,3}$ IgG were purchased from Genzyme (Cambridge, MA, cat. no. 1835-01, and 1294-01). Immulon I microtiter plates and wells were purchased from Fischer Scientific (Ottawa, ON). Crystalline bovine serum albumin (BSA), alkaline phosphatase-conjugated rabbit anti-chicken IgG, and alkaline phosphatase substrate (p-nitrophenylphosphate disodium) were obtained from Sigma Chemical co. (St. Louis, MO, USA, cat. no. A-4378, A-9171, and P-104). Chicken anti-TGF-ß neutralizing antibody was obtained from R&D Systems, Inc. (Minneapolis, MN, USA, cat. no. AB-101-NA).

Patients

Seven thermally-injured adult male patients who were receiving their acute and rehabilitative burn care at the University of Alberta burn treatment unit and outpatient clinic were selected for the study. Only those individuals

exhibiting red, raised, puritic, and painful scars confined to the region of injury that affected greater than 5% total body surface area were considered for the interferon trial, after providing informed consent (Table 4-1). The study protocol was approved by the Research Ethics Committee of the University of Alberta Hospitals.

Phase I/II Interferon Trial

After initial physical examination and complete blood count, urinalysis, thyroid, liver and renal functional panels, consenting patients underwent standardized photography of their hypertrophic scar regions. Patients were followed monthly with physical examination, scar volume, and scar assessment measurements. After eight weeks and three examinations patients and/or their spouses were taught self-administration of IFN-α2b subcutaneously by a research nurse. Two million units of human recombinant IFN-α2b were administered subcutaneously daily for seven days by the patient or their spouse under the supervision of a research nurse in the outpatient clinic. Blood pressure, pulse, respiratory rate, oricular temperature, and response to injection were recorded. Thereafter, $3X10^6$ units were administered three times per week. Records were kept of used interferon vials and systemic response to injection in an attempt to determine compliance with therapy. Serial serum samples were collected every month in the early afternoon over the course of treatment and one month after the conclusion of therapy.

Serum Collection

Human serum was obtained from IFN-α2b-treated patients and a control population (generously provided by the Canadian Red Cross). Venous blood samples were taken in collection vials without the presence of an anticoagulant. The samples were allowed to clot, and then centrifuged at 4°C and 3,000 rpm for 30 minutes. The supernatant was then aliquotted into 1.5 ml Eppendorf tubes and stored at -80°C until use.

Acid-Ethanol Extraction of total TGF-ß from Serum

Total TGF-ß (both active and latent) was extracted from serum as described by Roberts et al. (1980). Briefly, 0.5 ml serum samples were diluted 1:1 with distilled water, vortexed, and then acidified with 4.0 ml of acid-ethanol solution (93% ethanol, 2% concentrated HCl (0.24M), 85 mg/L PMSF, 5 mg/L Pepstatin A). The samples were then vertically rotated overnight at 4°C, and centrifuged at 10,000Xg for 30 minutes. The supernatants were collected and stored at 4°C, and the pellets were re-extracted by another overnight vertical rotation at 4°C in 4.0 ml of acid-ethanol solution. After re-centrifugation at 10,000 X g for 30 minutes, the pellets were discarded, the two supernatants were then combined and adjusted to pH 5.2 with12M ammonium hydroxide. The samples were buffered with 2M ammonium acetate pH 5.3 (1.0 ml for every 85 ml of supernatant), and diluted 1:3 with cold 99% ethanol (-20°C). The solutions were precipitated at -20°C for 48 hours, and then centrifuged at 10,000 X g for 30 minutes. The supernatants were discarded, the pellets dissolved in 4.0 ml of 1M acetic acid, and dialyzed against 1% acetic acid in Spectrapore dialysis tubing (6000-8000 M.W.-cutoff). The samples were then frozen at -20°C, and lyophilized to yield a partially purified TGF-ß product.

SELISA for TGF-ß1

The TGF-ß SELISA was modified from the methodology of Danielpour (1993). Immulon I microtiter wells were coated with 100 µl/well of a mouse monoclonal anti-TGF-ß$_{1,2,3}$ primary antibody diluted in Dulbecco's phosphate buffered saline (PBS), incubated at room temperature for 60 minutes, and then overnight at 4°C. The wells were then warmed to room temperature for 20 minutes, the antibody solution was discarded, and the wells washed once (200 µl/well) with a PBS/0.05% Tween 20 wash solution (WB). The wells were then blocked for 60 minutes(175 µl/well) with 1% crystalline BSA solution in Tris buffered saline (150 mM NaCl, 100 mM Tris, pH 7.6). Stock ultrapure human TGF-ß$_1$ in sterile PBS (20 mg/ml) was then diluted 1:160 in BB1 assay buffer (0.1% crystalline BSA, 0.1% Tween 20, 0.02% sodium azide, 150 mM NaCl, 100 mM Tris, pH 7.6) to give a diluted stock concentration of 0.125 ng/ml. The diluted stock concentration was further

diluted in BB1 to give standard concentrations for the assay (0-250 pg/100ml). Lyophilized experimental samples were also diluted (1:720) in BB1 assay buffer, and then both experimental and standard TGF-ß samples were incubated in ELISA wells (100 µl/well) for 60 minutes. Both standard and experimental samples were incubated in triplicate. Following 3 washes (200 µl/well) with WB (5 minutes each), the wells were incubated (100 µl/well) with a 1:200 dilution of the stock concentration of chicken-anti-TGF-ß polyclonal antibody (1 mg/ml) for 60 minutes. The wells were then washed 5 times (200 µl/well) with WB (5 minutes each), and incubated for 60 minutes (100 µl/well) with a 1:3000 dilution of the stock concentration of rabbit-anti-chicken IgG alkaline phosphatase. After 5 more washes (200 µl/well) with WB (5 minutes each), the wells were incubated (100 µl/well) with a 1 mg/ml concentration of p-nitrophenylphosphate in 1.0 M diethanolamine buffer (pH 9.8). Colour was allowed to develop for 1-1.5 hours at room temperature, and then the wells were read on an automated spectrophotometric plate reader (EAR400AT, SLT Instruments, Austria) at 405 nm. For quantitation of unknown concentrations of TGF-ß$_1$, triplicate experimental data points were analyzed by linear regression analysis and interpolation from the standard curve.

Statistics

Statistical comparisons between HSc patients and the control group were made with an unpaired students t-test. Multiple comparisons within the patient population data over the IFN-α2b treatment period were done by multiple analysis of variance (MANOVA), using the Boneferroni correction for repeated measures. P values <0.05 were considered significant.

RESULTS

HSc Patient and Control Group Serum TGF-ß$_1$ Levels

Serum TGF-ß$_1$ levels in a control population of twenty-six healthy male blood donors was determined by SELISA based on the methodology of Danielpour (1993). The ages of the control group ranged from 19-66 years, with a mean age of 41.6 years. The serum TGF-ß$_1$ concentrations ranged

between 43.64±0.73 ng/ml and 74.31±4.63 ng/ml, with the population mean occurring at 56.85±8.38 ng/ml (Figure 4-1; Table 4-2). No age related variation in serum TGF-ß1 levels was observed in the control population.

Serum samples from seven patients with severe post-burn HSc (1-3 samples/patient, measured in triplicate) displayed significantly higher TGF-ß1 concentrations than those from the sex-matched control group (Figure 4-2). Though the ages of the patients and their degree of injury varied considerably (Table 4-1), all samples tested displayed TGF-ß1 levels greater than the mean concentration of the control population (Table 4-2).

Patient Serum TGF-ß1 Levels During IFN-α2b Treatment

Systemic administration of IFN-α2b ($3x10^6$ units, three times/week) for six months, following an eight week control period (see Materials and Methods), significantly decreased total serum TGF-ß1 levels in patients with HSc. A typical patient profile is shown in Figure 4-3. Upon initiation of treatment, an immediate reduction in serum levels is observed within one month, and this effect appears to persist throughout the course of treatment. Analysis of the entire data set reveals a similar phenomenon (Figure 4-4; Table 4-2). After three months of treatment, mean serum TGF-ß1 levels in the patient population reduced significantly from pre-treatment levels. After a further three months of treatment, serum levels remained significantly reduced from pre-treatment levels. Four patients were followed-up one month post treatment, and their serum levels remained significantly reduced from pre-treatment levels. No significant differences were observed among early-treatment, late-treatment, post-treatment, and control groups.

DISCUSSION

These results strongly suggest a role for circulating TGF-ß1 in the development of post-burn HSc. Significantly higher levels of serum TGF-ß are observed in patients with HSc compared to an age and sex-matched control population. Systemic treatment with IFN-α2b results in the reduction of patient serum TGF-ß into the normal range with coincident scar resolution and clinical improvement (data not shown).

Several caveats, however, apply to these findings. As the acid-ethanol extraction procedure results in the partial purification and activation of total circulating TGF-ß, it is unclear whether the differences reflected in the data are due to changes in platelet TGF-ß levels or plasma TGF-ß levels; however, both situations could significantly affect the quality of wound healing (Roberts et al., 1980; Grainger et al., 1995a). Also, the fraction of total TGF-ß that is active in the circulating pool cannot be determined by this procedure, though the biological functions of active TGF-ß in serum have not been well elucidated.

While several groups have correlated concentrations of circulating TGF-ß with the onset and progression of pathological conditions, little work has been done on the *in vivo* antagonism of the growth factor (Anscher et al., 1993; Snowden et al., 1994). Studies done with the systemic administration of the proteoglycan decorin suggest that *in vivo* antagonism of TGF-ß is an effective measure against the progression of renal fibrosis (Border et al., 1992). Conversely, the use of aspirin has been shown to increase serum concentrations of active TGF-ß and to inhibit the progression of artherosclerosis (Grainger et al., 1995b). In this study, IFN-α2b seems to downregulate significantly serum concentrations of TGF-ß as it augments the resolution of HSc.

This study, therefore, provides evidence that HSc may be the result of a systemic condition characterized by the impaired regulation of circulating TGF-ß. Further, it suggests that antagonism of TGF-ß may be the key to the clinical management of HSc.

Table 4-1.

IFN-α2b Phase I/II Clinical Trial Patient Data

Patient	Age (Years)	Sex	TBSA (%)	Treatment Period (months post-injury)
1. CF	49	Male	30	17
2. DM	31	Male	60	20
3. RS	49	Male	32	17
4. GG	26	Male	70	20
5. LM	36	Male	65	4
6. ESa	29	Male	85	8
7. ESt	25	Male	45	6
Mean	36.4±3.6		50.9±8.0	13.5±2.3

Table 4-1. Seven male patients between the ages of 25 and 49 years were used in a Phase I/II clinical trial of recombinant human IFN-α2b for the management of post-burn HSc. The total burn surface area (TBSA) experienced by the patients ranged between 32-85%, and initiation of treatment occurred at various stages post-injury.

Table 4-2.

Serum TGF-β_1 from HSc patients and Control Group

Category	Serum TGF-β_1 (ng/ml) (mean±S.D.)	Range (ng/ml) (mean±S.D.)
Control (n=26)	56.85±8.38*	43.64±0.73 - 74.31±4.63
Pre-Rx (n=14)	123.04±36.48	69.42±4.87 - 232.93±7.92
Early-Rx (n=21) (0-3 months)	66.17±22.37†	36.11±9.92 - 120.41±2.89
Late-Rx (n=18) (4-6 months)	63.65±22.37†	8.51±1.24 - 106.99±9.51
Post-Rx (n=4) (7 months)	54.57±20.48†	25.60±9.34 - 74.63±4.11

* Significantly different from pre-Rx by unpaired t-test ($p<0.05$)

† Significantly different from pre-Rx by MANOVA ($p<0.05$)

Table 4-2. Serum TGF-β_1 levels from patients with post-burn HSc before treatment with IFN-α2b (Pre-Rx) levels are significantly higher than levels after three months of treatment (Early-Rx) and after six months of treatment (Late-Rx). One month post-treatment (Post-Rx) serum levels remain significantly different from pre-Rx levels. No significant differences were found among Early-Rx, Late-Rx, Post-Rx, and Control groups.

Figure 4-1.

Scatterplot of Control Group serum TGF-ß1 levels

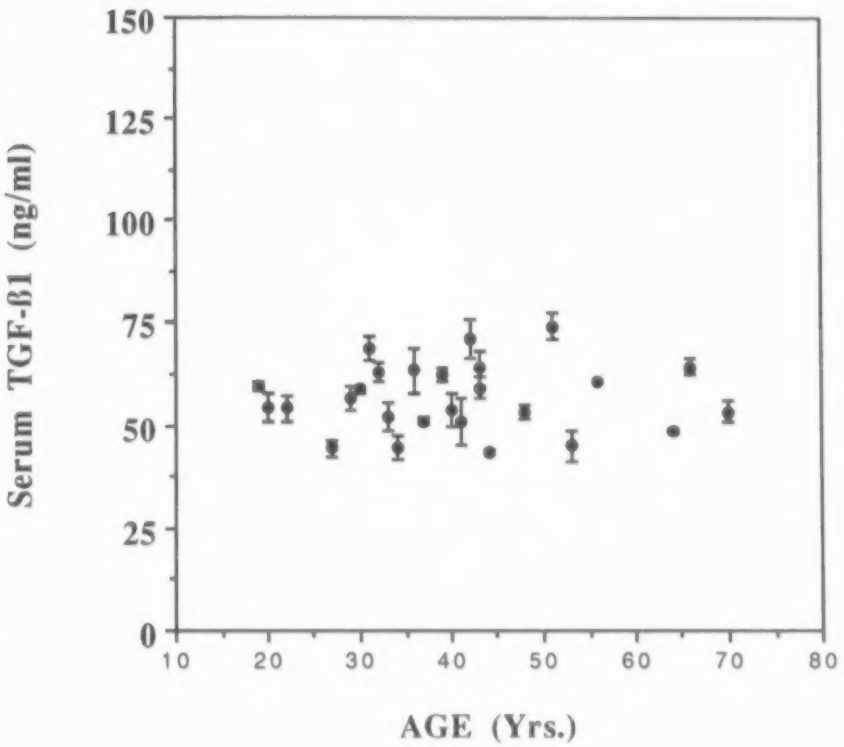

Figure 4-1. Analysis of serum TGF-ß1 levels from twenty-six healthy male blood donors revealed no age-related fluctuations. The population displayed a tight distribution around the mean of 56.85±8.38 ng/ml.

Figure 4-2.

Scatterplot of HSc patient and Control Group serum TGF-ß1 levels

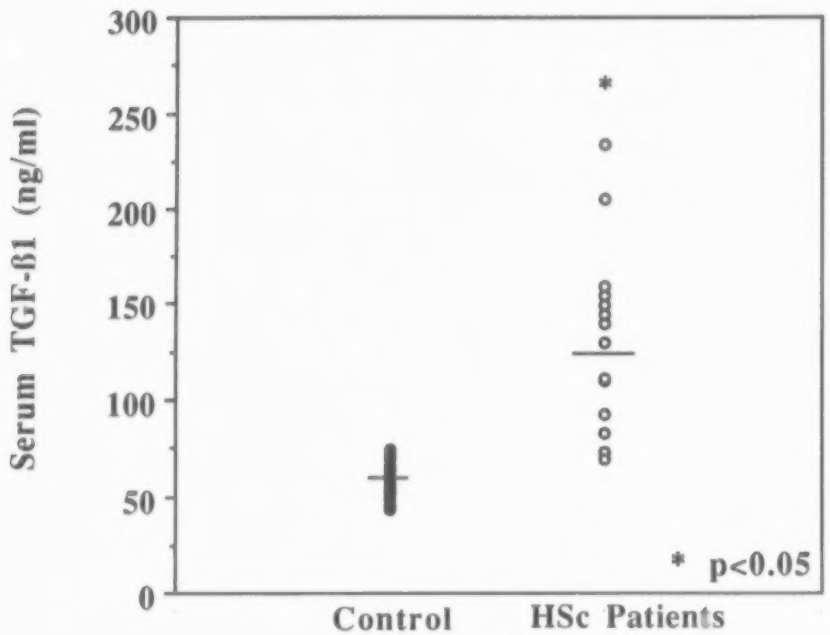

Figure 4-2. Comparison of the scatterplots of serum TGF-ß1 levels demonstrates significant elevation of HSc levels relative to controls.

Figure 4-3.

Longitudinal study of serum TGF-ß1 levels in one patient during treatment with IFN-α2b

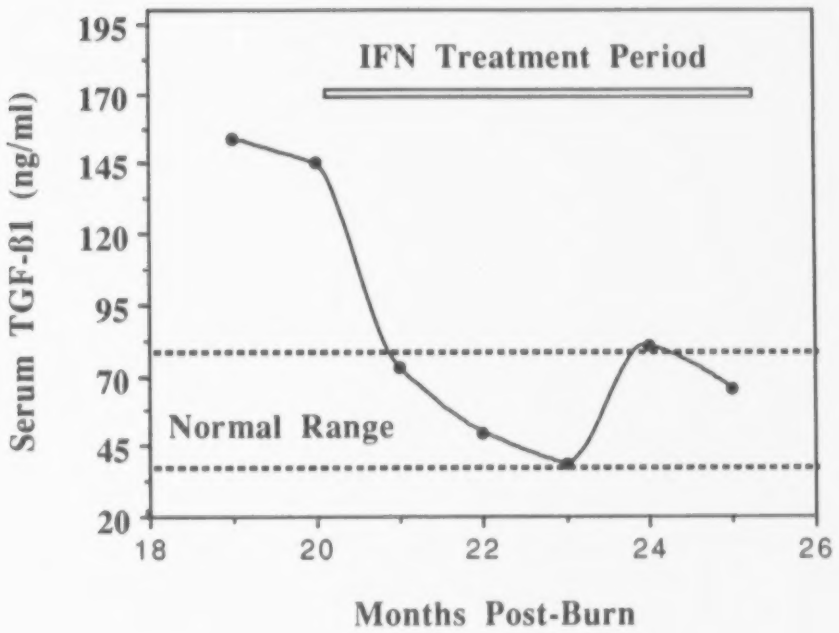

Figure 4-3. Data from patient RS demonstrates that IFN-α2b reduces serum TGF-ß1 levels into the normal range, and has a persistent effect over the duration of the treatment period.

Figure 4-4.

Scatterplots of HSc patient serum TGF-ß1 levels over the course of IFN-α2b treatment

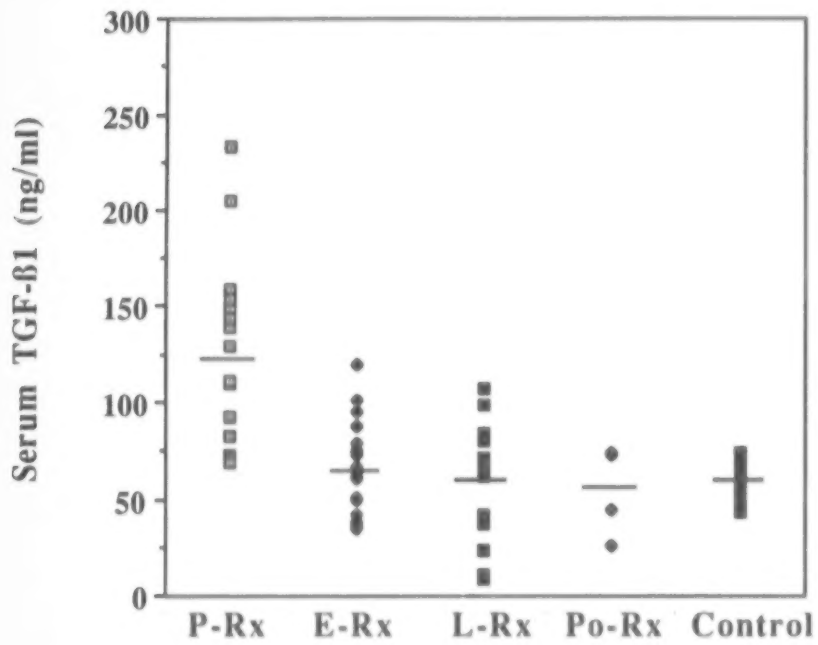

Figure 4-4. IFN-α2b reduces high pre-treatment (P-Rx) serum TGF-ß1 levels in all patients into the normal range (Control) within three months (E-Rx). The effect persists through to six months of treatment (L-Rx), and in four patients up to one month post-treatment (Po-Rx).

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CHAPTER 5 - EFFECTS OF IFN-α2b AND IFN-γ ON TGF-ß SECRETION BY HYPERTROPHIC AND NORMAL DERMAL FIBROBLASTS *IN VITRO*

INTRODUCTION

Hypertrophic scarring (HSc) and keloids are fibroproliferative disorders of human skin (Rockwell et al., 1989). They are characterized by the development of raised, erythematous, and non-pliable scar tissue following thermal or other forms of traumatic injury that penetrate the dermis (Robb et al., 1987). While generally not life-threatening, these scars have a bulky and inelastic quality that often compromises cosmetic appearance and limits the mobility of joints. Presently available therapies for these disorders include the use of pressure garments and silicone gel sheets, the intra-lesional injection of corticosteroids, and surgical revision. While providing some relief for afflicted individuals, none of these treatments are completely satisfactory (Scott et al., 1994).

Histological analysis of HSc biopsies demonstrates excessive and disorderly production of extracellular matrix components, mainly collagen, during the wound healing process (Uitto et al., 1986). Explantation and culture of fibroblasts from HSc and normal tissues has revealed differential expression of type I and type III collagen mRNA and protein by burn fibroblasts, whereby production of type I collagen was markedly increased relative to normal controls, and type III collagen was significantly reduced (Ghahary et al., 1992).

While the etiology of HSc and keloid formation is not well established, it has been suggested that persistence of the cytokine transforming growth factor-ß (TGF-ß) during the healing process may contribute to the development of these pathologies. Several studies have demonstrated that TGF-ß is mitogenic for dermal fibroblasts, and promotes the synthesis of extracellular matrix proteins, including collagen (Ignotz and Massagué, 1986; Peltonen et al., 1990; Varga et al., 1987). Antibodies to TGF-ß can inhibit scar formation in mice, while addition of TGF-ß to healing wounds has been shown to augment scar formation (Shah et al., 1992). Our previous work has shown elevated levels of TGF-$ß_1$ mRNA and protein present in biopsy sections of hypertrophic scars compared to the normal dermis of the same patient

(Ghahary et al., 1993; Scott et al., 1995). Evidence in this thesis demonstrates that serum levels of TGF-ß1 are elevated in HSc patients compared to an age and sex-matched control population. Antagonism of TGF-ß production, therfore, might be an important means by which HSc can be regulated.

Interferon-α and interferon-γ (IFN-α and IFN-γ) are two cytokines that have been shown to possess antiproliferative and anti-fibrogenic effects on fibroblasts *in vitro*. Studies on fibroblasts from pulmonary fibrosis, scleroderma, Peyronie's disease, keloids, and HSc have demonstrated that the interferons inhibit cell division and reduce matrix synthesis (Duncan and Berman., 1987; Berman and Duncan 1989; Duncan et al., 1991; Tredget et al., 1993). *In vivo*, IFN-γ has been shown able to inhibit fibrosis to implanted foreign bodies, and reduce type I collagen expression in an animal model of hepatic fibrosis (Granstein et al., 1987; Czaja et al., 1989). Furthermore, the use of intralesionally administered IFN-α2b and IFN-γ have proven useful in the clinical management of keloids and HSc (Berman and Duncan, 1987; Pittet et al., 1994).

While the effects of the interferons on fibroblast proliferation and the production of collagen have been studied extensively, their effects on fibroblast TGF-ß production have not. Evidence in this thesis suggests that IFN-α2b applied systemically reduces serum TGF-ß1 levels in patients with HSc as it facilitates the scar resolution. Furthermore, the interferons can inhibit contraction of fibroblast-populated collagen lattices, a phenomenon thought to be mediated by TGF-ß (Dans and Rivkah, 1994; Nedelec et al., in press). It is not clear, however, whether the actions of systemically administered interferons reduce TGF-ß production by altering local levels directly, or rather by initiating a cascade of effects that results in the local or systemic antagonism of TGF-ß production. The purpose of this study is to determine if TGF-ß is differentially produced by hypertrophic and normal skin fibroblasts, and to examine the effects of IFN-γ and -α2b on fibroblast TGF-ß secretion *in vitro*.

MATERIALS AND METHODS

Materials

Recombinant human interferon-γ was purchased from Hoffmann-La Roche inc. (Nutley, NJ, USA). Human recombinant Intron A^R was obtained from Schering (Pointe Claire, Quebec). Cell culture flasks, 6- and 24-well cell culture plates, and cryopreservation tubes were purchased from Corning (New York, NY, USA). Fetal bovine serum (FBS), medium antibiotic/antimycotic preparation, and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco (Grand Island, NY, USA). Ultrapure natural human TGF-ß1 and mouse monoclonal anti-TGF-$ß_{1,2,3}$ IgG were purchased from Genzyme (Cambridge, MA, cat. no. 1835-01, and 1294-01). Immulon I microtiter plates and wells were purchased from Fischer Scientific (Ottawa, ON). Dimethyl sulfoxide (DMSO), 97% pure crystalline bovine serum albumin (BSA, cat. no. A-4378), alkaline phosphatase-conjugated rabbit anti-chicken IgG (cat. no. A-9171), and alkaline phosphatase substrate (p-nitrophenylphosphate disodium, cat. no. P-104) were obtained from Sigma Chemical co. (St. Louis, MO, USA). Chicken anti-TGF-ß neutralizing antibody (cat. no. AB-101-NA), were obtained from R&D Systems, Inc. (Minneapolis, MN, USA).

Clinical Specimens

Specimens for this study were obtained from three patients seen at the University of Alberta Hospitals with HSc development subsequent to cutaneous thermal injury (Table 5-1). All patients demonstrated raised, erythematous, pruritic, and non-compliant scars confined to the site of injury. The patients, all males, ranged in ages from 4 to 32 years. After informed consent, small pieces of both HSc and the normal dermis of the same patient were obtained during required surgical revision procedures.

Paired cell strain Explantation and Culture

Fibroblast cultures were prepared from clinical specimens according to the methods described by Nakano and Scott (1986). Briefly, clinical specimens were collected during surgery and quickly immersed in sterile DMEM

supplemented with FBS (10%), and an antibiotic/antimycotic preparation (Ab, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B). Specimens were then dissected free of fat and minced into small pieces less than 0.5 mm in diameter, washed with DMEM, and distributed (six pieces/ per flask) into 25 cm^2 cell culture flasks. The pieces were then incubated in DMEM/Ab/50% FBS for three days, DMEM/Ab/25% FBS for three days, and DMEM/Ab/10% FBS for several weeks in an atmosphere of 5% CO_2 in air at 95% relative humidity and 37°C. Medium was replaced weekly after the first six days for approximately four weeks, at which point fibroblast outgrowth from the specimens had exceeded 50% of the growth surface. At this time the fibroblasts were released from the culture flasks by brief treatment with 0.25% trypsin/EDTA in PBS and subsequently seeded into 75 cm^2 culture flasks in DMEM/Ab/10% FBS. Upon reaching confluence, the cells were released by trypsinization, split for a subculture of 1:6 and re-seeded into 75 cm^2 culture flasks. Cells were then trysinized again at confluence, suspended (2.5 X 10^6 cells/ml) in freezing medium (DMEM/Ab/10%FBS/5%DMSO), aliqotted into cryopreservation tubes, and stored in liquid nitrogen until use. Cell lines at passage 5 were used in all experiments (Table 5-1).

Treatment of Fibroblasts for total TGF-ß analysis

Both HSc and patient-matched normal dermal fibroblasts were seeded into either 6 or 24-well cell culture flasks and grown to visual confluence in DMEM/Ab/10% FBS. The medium of the control cells was then replaced with DMEM/Ab/2% FBS, while the experimental cell medium was changed to DMEM/Ab/2% FBS supplemented with either IFN-γ (1000 U/ml) or IFN-α2b (2000 U/ml) according to the findings of Harrop et al. (1995) and Tredget et al. (1993). The cells were cultured for 72 hours, at which point the media was changed to DMEM/Ab/0.5% FBS, with experimental groups retaining treatment concentrations of IFN-α2b and IFN-γ. After a further 48 hours in culture, media was harvested from wells for TGF-ß ELISA analysis. The cells were released by trypsinization, and counted using a Coulter counter (Coulter Electronics, Hialeah, FL, USA).

Activation of Latent TGF-ß

Total latent TGF-ß was activated in conditioned medium samples by mild acidification (Grainger et al., 1995). Briefly, 0.2 ml of 1N hydrochloric acid was added to 1.0 ml of conditioned medium to give a final concentration of 0.167 N HCl. The solutions were vortexed and incubated for 10 minutes at room temperature. The medium was then neutralized with 0.2 ml of 1.2N NaOH/0.5 M Hepes free acid to give a pH between 7.2 and 7.6. The samples were then assayed directly as described below.

SELISA for TGF-ß

The TGF-ß SELISA was modified from the methodology of Danielpour (1993). Immulon I microtiter wells were coated with 100 µl/well of a mouse monoclonal anti-TGF-ß$_{1,2,3}$ primary antibody diluted in Dulbecco's phosphate buffered saline (PBS), incubated at room temperature for 60 minutes, and then overnight at 4°C. The wells were then warmed to room temperature for 20 minutes, the antibody solution was discarded, and the wells washed once (200 µl/well) with a PBS/0.05% Tween 20 wash solution (WB). The wells were then blocked for 60 minutes(175 µl/well) with 1% crystalline BSA solution in Tris buffered saline (150 mM NaCl, 100 mM Tris, pH 7.6). Stock ultrapure human TGF-ß$_1$ in sterile PBS (20 mg/ml) was then diluted 1:160 in BB1 assay buffer (0.1% crystalline BSA, 0.1% Tween 20, 0.02% sodium azide, 150 mM NaCl, 100 mM Tris, pH 7.6) to give a diluted stock concentration of 0.125 ng/ml. The diluted stock concentration was further diluted in BB1 to give standard concentrations for the assay (0-250 pg/100ml). Lyophilized experimental samples were also diluted (1:720) in BB1 assay buffer, and then both experimental and standard TGF-ß samples were incubated in ELISA wells (100 µl/well) for 60 minutes. Both standard and experimental samples were incubated in triplicate. Following 3 washes (200 µl/well) with WB (5 minutes each), the wells were incubated (100 µl/well) with a 1:200 dilution of the stock concentration of chicken-anti-TGF-ß polyclonal antibody (1 mg/ml) for 60 minutes. The wells were then washed 5 times (200 µl/well) with WB (5 minutes each), and incubated for 60 minutes (100 µl/well) with a 1:3000 dilution of the stock concentration of rabbit-anti-chicken IgG alkaline phosphatase. After 5 more washes (200 µl/well) with

WB (5 minutes each), the wells were incubated (100 µl/well) with a 1 mg/ml concentration of p-nitrophenylphosphate in 1.0 M diethanolamine buffer (pH 9.8). Colour was allowed to develop for 1-1.5 hours at room temperature, and then the wells were read on an automated spectrophotometric plate reader (Molecular Devices Corporation, USA) at 405 nm. For quantitation of unknown concentrations of TGF-ß, triplicate experimental data points were analyzed by linear regression analysis and interpolation from the standard curve.

Statistics

Students paired t-tests were used to compare treatment groups, control groups, and paired strains. P values < 0.05 were considered significant.

RESULTS

Effects of IFN-α2b and IFN-γ on TGF-ß secretion in paired cell strains

Comparison of total TGF-ß production by three confluent HSc and normal strains after 48 hours in culture with DMEM/0.5% FBS revealed that hypertrophic cells secrete significantly more TGF-ß than patient-matched normal cell strains (Figure 5-1; Table 5-2).

When the HSc cells were treated with IFN-α2b, a statistically significant reduction in secreted TGF-ß levels was observed to levels consistant with those of normal cells. A reduction in TGF-ß production was observed when normal cells were treated with IFN-α2b, though the difference was not statistically significant (Figure 5-2; Table 5-2).

Treatment of HSc and normal cells with IFN-γ also demonstrated a reduction in fibroblast TGF-ß secretion, though these were not statistically significant. Interferon-γ did, however, reduce TGF-ß levels in hypertrophic cells to levels similar to those of untreated normal cells (Figure 5-3; Table 5-2).

DISCUSSION

The antagonism of TGF-ß secretion may be an important means to inhibit the progression of fibrosis . A number of studies have demonstrated that *in vitro* and *in vivo* antagonism of TGF-ß by soluble macromolecules significantly alters the cellular response to TGF-ß and can reverse the process of disease (Yamaguchi et al., 1990; Border et al., 1992). This data demonstrates that TGF-ß production by human HSc fibroblasts is significantly higher than by normal patient-matched human fibroblasts. Further, treatment with IFN-α2b significantly reduces TGF-ß secretion by HSc cells to levels within the normal range.

Interestingly, this data suggests that IFN-γ may also reduce TGF-ß secretion by fibroblasts in culture, and while no sigificant effects were observed, increasing numbers of experiments might change the statistical relationships among the experimental groups.

It is not clear whether the inhibitory effects of the interferons on fibroblast TGF-ß production are due to direct or indirect mechanisms. The fact that reductions in TGF-ß secretion are observed only after prolonged incubation suggests that indirect mechanisms, possibly the activation of paracrine pathways, are responsible for the observed effects. Further studies involving the blockage of *de novo* protein synthesis immediately following treatment can clairify this issue.

While this study demonstrates that fibroblast treatment with the interferons reduces amounts of secreted TGF-ß, further examination of mRNA levels and intracellular protein trafficking must be done to determine if TGF-ß production is in fact altered. The *in vitro* data, however, supports the *in vivo* observations in Chapter Four of this thesis, and suggests that the clinical efficacy of IFN-α2b in the management of HSc may be in part due to its ability to inhibit the cellular secretion of TGF-ß.

Table 5-1.

Cell strain data

Cell Strain	Sex	Age (yrs.)	TBSA (%)	Anatomical Site (H/N)	TPI (mos.)
H8/N8	M	32	60	r-chest/r-chest	6
H5/N5	M	4	32	bi-hands/STSG	5
H2/N2	F	28	4	r-arm/r-arm	14

Table 5-1. Cells were obtained from three patients of various ages. The total burn surface area of each patient were very different (TBSA), as were the anatomical sites, and the time post-injury that the cells were explanted (TPI). When a matched anatomical site could not be harvested for normal skin, skin was harvested from a split-thickness skin graft (STSG).

Table 5-2.

Cell treatment data

Treatment Group (cells/treatment)	TGF-ß secretion (pg/ml/10^4 cells)	Number (n)
HSc/ Untreated	71.50± 20.86	7
HSc/IFN-α2b	27.49± 6.91*	7
HSc/IFN-γ	38.91± 4.76	7
Normal/Untreated	41.05± 18.13*	7
Normal/IFN-α2b	38.22± 10.63	7
Normal/IFN-γ	10.24± 3.77	7

* Significantly different from HSc/Untreated by paired t-test ($p<0.05$)

Table 5-2. TGF-ß secretion by HSc fibroblasts is significantly different from that of normal skin fibroblasts of the same patient (mean± S.E.M). Treatment with either IFN-α2b and IFN-γ causes a reduction in TGF-ß levels to that of the normal controls.

Figure 5-1.

TGF-ß secretion by HSc and normal dermal fibroblasts

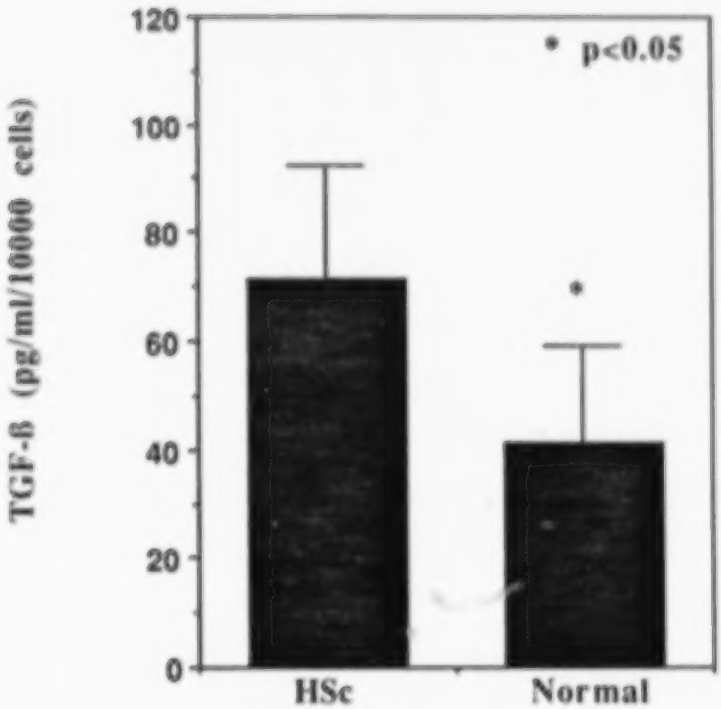

Figure 5-1. TGF-ß secretion by HSc fibroblasts is significantly higher than by patient-matched normal skin fibroblasts.

Figure 5-2.

Effect of IFN-α2b on TGF-ß secretion by normal and HSc fibroblasts

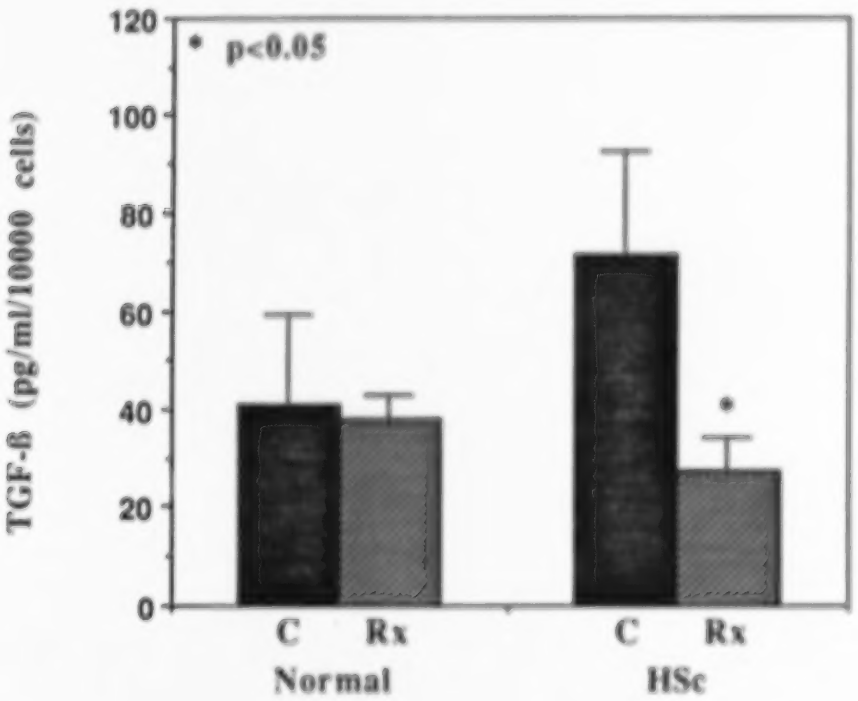

Figure 5-2. Treatment of HSc fibroblasts with IFN-α2b (HSc Rx) significantly reduces TGF-ß secretion to levels similar to normal controls (Normal C). Treatment of normal fibroblasts with IFN-α2b (Normal Rx) causes no significant reduction in TGF-ß secretion relative to Normal C.

* Significantly different from HSc C by paired t-test ($p<0.05$)

Figure 5-3.

Effect of IFN-γ on TGF-ß secretion by Normal and HSc Fibroblasts

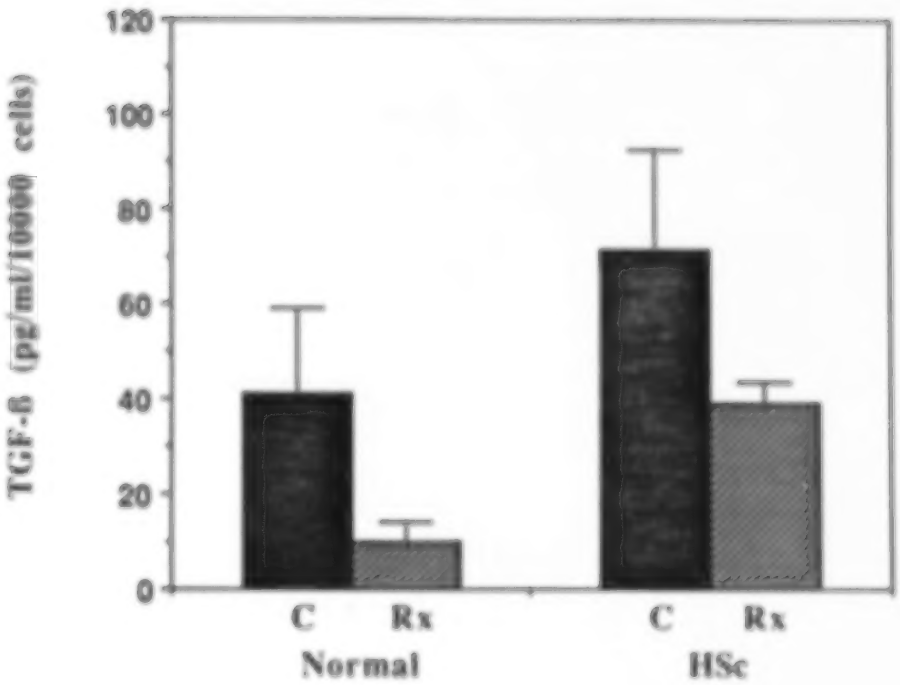

Figure 5-3. Treatment of Normal and HSc fibroblasts with IFN-γ (Normal Rx, HSc Rx) does not significantly reduce secreted TGF-ß levels compared to strain controls (Normal C, HSc C).

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CHAPTER 6 - GENERAL DISCUSSION

A. INTRODUCTION

It is well established that most if not all of the ordered processes that result in wound healing are under the control of systemically and locally secreted bioactive molecules termed cytokines. As these molecules are the signals that coordinate the various phases of tissue repair, it has been suggested that a breakdown in the regulation of growth factor production can lead to the development of wound healing pathologies (Scott et al., 1994).

Transforming growth factor-ß (TGF-ß) is a cytokine that has been linked to the development of numerous wound healing disorders. It is ubiquitously expressed, fibrogenic and mitogenic for mesenchymal cells, and an important regulatory molecule in the wound healing cascade (Roberts and Sporn, 1990). While the mechanisms by which it causes disease are unclear, TGF-ß has been implicated as a causative agent in the development of numerous types of pathological fibrosis.

Hypertrophic scarring (HSc) may be one such pathology. It arises in wounds where there is an imbalance in extracellular matrix metabolism. The equilibrium between collagen synthesis and degradation is upset, ultimately resulting in the overabundant accumulation of matrix components upon healing (Uitto et al., 1986; Rockwell et al., 1989). High levels of TGF-ß mRNA and protein have been correlated with the development of this condition (Ghahary et al., 1993; Scott et al., 1995), The hypothesis of this thesis is that systemic and local disregulation in the production of TGF-ß is the key to the development of HSc, and that antagonism of TGF-ß production by the theraputic administration of IFN-α2b may be the means by which HSc can be controlled.

B. SYNOPSIS OF RESULTS

The results of this study suggest a great deal about the etiology of HSc. When measured by a SELISA based on Danielpour's methodology (1993), serum TGF-ß levels in patients with severe post-burn HSc were significantly higher than a control group of age and sex matched healthy individuals. Upon treatment with systemically administered IFN-α2b, in a phase I/II clinical

trial, reductions in serum TGF-ß levels into the normal range were observed within one month of treatment, and persisted up to one month post-treatment period, coincident with significant clinical improvements and scar resolution.

Furthermore, in an *in vitro* study, fibroblasts explanted from HSc at various stages post-injury demonstrated significantly higher secretion of TGF-ß compared to simultaneously explanted fibroblasts derived from the normal skin of the same patient. Upon treatment with either IFN-α2b or IFN-γ, TGF-ß secretion by these cells was reduced to levels approximating those of the normal control group, reflecting the results of the *in vivo* study. Treatment of normal cells with either cytokine did not significantly downregulate TGF-ß secretion.

The general implications of these results are threefold. They suggest that there may be a systemic component to HSc, as serum TGF-ß levels in patients with the condition were significantly higher than controls. They suggest that antagonism of TGF-ß production by IFN-α2b *in vivo* may be a means by which this pathology can be regulated. Lastly, they suggest that fibroblasts explanted from HSc have different functional characteristics compared to normal fibroblasts of the same individual.

There are several limitations to this study. First, the biological significance of high levels of TGF-ß in serum has not been determined. As acid-ethanol extraction does not discriminate between active and latent TGF-ß, the relevance of high serum concentrations of TGF-ß is not clear. Secondly, though the healthy male control group was useful to demonstrate differences between HSc patients and a normal sex- and age-matched population, it did not control for differences between HSc patients and the burn population. As all the patients in the study had experienced serious burn injuries, it is important to recognize that high serum TGF-ß levels may simply be a consequence of that fact. Furthermore, a blinded and controlled phase III study using systemically administered IFN-α2b must be done to ensure that the effects of IFN-α2b on HSc resolution and TGF-ß production are due to the treatment. Without a control group receiving a placebo, it is difficult to make any definitive statements concerning the efficacy of the procedure. Finally, the use of an *in vitro* cell culture system, even on paired cell strains, is of questionable biological significance. The system represents a serious departure from any biologically relevant conditions, and is subject to numerous uncontrollable artifacts.

D. SUMMARY AND FUTURE WORK

There is tremendous potential for future work in this area. Firstly, use of a receptor-based SELISA system may be useful in the discrimination of active and latent pools of TGF-ß present in serum, as recent evidence has correlated levels of the active form in serum with disease (Grainger et al., 1995). Also, analysis of serum TGF-ß levels in a control group of burn patients without HSc would further characterize the relationship between serum TGF-ß and HSc development. The *in vitro* data suggests heterogeneity in fibroblast populations. Though extremely controversial, this idea can be examined further through genetic testing and functional characterization of explanted fibroblasts, It will be necessary, however, to manipulate the culture conditions in order to better reflect the *in vivo* environment.

This thesis demonstrates that there is a strong scientific basis for the clinical benefits observed in the use of interferon therapy for HSc. Improved drug delivery techniques and studies on the timing and dose of treatment may ultimately provide adequate relief for patients afflicted with this disorder.

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